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The role of β_1 Pix/caveolin-1 interaction in endothelin signaling through G α subunits

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Abstract

Endothelin-1 (ET-1) is a potent mitogen that transmits signals through its cognate G protein-coupled receptors to stimulate extracellular signal-regulated kinase Erk1/2. Endothelin-1 receptors (ET-Rs) are known to interact with caveolin-1 and co-localize in caveolae which integrate different receptor and signaling proteins. We have recently shown that β_1 Pix binds specifically to ET-Rs. Here, we show that β_1 Pix binding to caveolin-1 is dependent on heterotrimeric G proteins activation state. β_1 Pix interaction with different G proteins is increased in the presence of the G protein activator AMF. Moreover, extraction of cholesterol with methyl- β -cyclodextrin disrupts the binding of β_1 Pix to G_{α_q} , $G_{\alpha_{12}}$ and phospho-Erk1/2 but not the binding of β_1 Pix to G_{β_1} . The disruption of β_1 Pix dimerization strongly reduced the binding of caveolin-1, G_{α_q} and $G_{\alpha_{12}}$. Constitutively active mutants of G_{α_q} and $G_{\alpha_{12}}$ increased Cdc42 activation when co-expressed with β_1 Pix but not in the presence of β_1 Pix dimerization deficient mutant β_1 Pix Δ (602–611). ET-1 stimulation increased the binding of phosphorylated Erk1/2 to β_1 Pix but not to β_1 Pix Δ (602–611). RGS3 decreased ET-1-induced Cdc42 activation. These results strongly suggest that the activation of ET-Rs leads to the compartmentalization and the binding of G_{α_q} to β_1 Pix in caveolae, where dimeric β_1 Pix acts as platform to facilitate the binding and the activation of Erk1/2.

Keywords

Caveolin-1; β_1 Pix; G_{α_q} ; $G_{\alpha_{12}}$; Endothelin; Cdc42; Caveolae

Introduction

Endothelin-1 (ET-1) is a potent mitogenic peptide that mediates a wide variety of cell signaling including cell proliferation and gene expression through its G protein-coupled receptors, ET_AR and ET_BR [1] which can activate different G proteins. ET_AR acts through G_q and G_s to stimulate phospholipase C (PLC) and adenylate cyclase respectively. ET_BR stimulates PLC via G_q , and inhibits adenylate cyclase through G_i [2;3]. ET receptors have also been shown to couple to G_{12} in NIH3T3 cells where G_{12} mediates the formation of actin stress fiber formation [4]. ET_AR and ET_BR are both localized within caveolae [5;6], invaginations of the plasma membrane containing caveolin proteins which act as scaffolding proteins to cluster different

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signaling molecules such as G_{α} subunits, Ha-Ras, PKC and Src [7]. We have shown that ET-1 induces cell proliferation through up-regulation of the Cdc42/Rac guanine nucleotide exchange factor β_1 Pix [8]. β_1 Pix contains a Src homology 3 (SH3) domain, a *Dbl* homology (DH) domain, a pleckstrin homology (PH) domain for activation of Rho GTPases and a leucine zipper (LZ) domain responsible for β_1 Pix dimerization [9]. ET-1 stimulation of mesangial cells induces β_1 Pix translocation to focal adhesion and Cdc42 activation through PKA-dependent phosphorylation of β_1 Pix [10]. Moreover, the activation of G_{α_s} signaling pathway results in the binding of 14-3-3 β to β_1 Pix through protein kinase A-dependent phosphorylation leading to the inhibition of Rac1 and membrane ruffle formation [11]. β_1 Pix regulates mast cell secretion through G_{α_i} -dependent mechanism [12] and β_1 Pix/ $G_{\alpha_{i3}}$ interaction mediates p66Shc activation induced by EGF receptor transactivation [13].

Although several studies have shown that Erk1/2 activation by extracellular stimuli requires small GTPases activation, the mechanisms whereby G protein coupled receptors (GPCRs) regulate this signaling pathway are not fully understood. One mechanism of Erk1/2 activation involves G_{α_i} -coupled receptor where ligand stimulation induces the release of $G_{\beta\gamma}$ leading to Ras activation. In addition, ET-1 can also activate Erk1/2 through EGF receptor transactivation [14]. In recent years, there has been increasing evidence that the activation of some small GTPases by GPCRs is mediated by the activation of guanine nucleotide exchange factors (GEFs). For example, a family of RhoAGEFs consisting of p115RhoGEF, PDZ-RhoGEF and leukemia-associated RhoGEF contain a regulator of G protein signaling homology domain that directly binds to $G_{\alpha_{12}}$ [15;16]. The p63RhoGEF, however, was found to link RhoA to $G_{\alpha_{q/11}}$ -coupled receptors by directly interacting with active $G_{\alpha_{q/11}}$ [17].

Materials and Methods

Materials

Cell culture media and supplements were obtained from Invitrogen (Carlsbad, CA). Endothelin-1 was purchased from Calbiochem (La Jolla, CA). Anti-ET_A-R and anti-ET_B-R were from Millipore (Billerica, MD). Anti-Myc, anti-caveolin-1, anti- $G_{\beta 1}$ and anti- G_{α} subunits antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA). Antiphospho-Erk1/2 was purchased from Cell Signaling (Danvers, MA). RGS3 antiserum was described previously [18].

Cell culture and transfection

Human mesangial cells were cultured in RPMI 1624 (Invitrogen) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μ g/ml) in a 37°C humidified incubator with 5% CO₂. Transient transfection of cells with mammalian expression vectors was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Plasmids

The Myc-tagged β_1 Pix and β_1 Pix Δ (602–611) plasmids have been described [8;11]. G_{α_q} , $G_{\alpha_{q209L}}$, $G_{\alpha_{12}}$, $G_{\alpha_{12Q231L}}$ were obtained from Missouri S&T cDNA Resource Center. RGS3 was inserted into pcDNA3.1 plasmid as described before [18].

Immunoprecipitation and Western blot analysis

Cells were transfected with the appropriate construct for 24 h. Cells were washed twice in phosphate-buffered saline (PBS) and lysed in lysis buffer containing 20 mM Tris, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1% Triton X-100, 1 mM sodium fluoride, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml pepstatin, and 1 μ g/ml leupeptin.

Equal amounts of proteins were separated by using 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore), immunoblotted with the appropriate antibody, and visualized by enhanced chemiluminescence (ECL; Amersham Biosciences, Inc.). For immunoprecipitation, antibodies against Myc, caveolin-1 or G α subunits were added to the cell lysate (500 μ g) for 2 h of incubation, followed by addition of protein A- or protein G-agarose beads for an additional hour. The beads were washed three times in PBS. The immunoprecipitated proteins were released from the beads by boiling in 1x sample buffer for 5 min and subsequently analyzed by Western blotting. Total cell lysate (TCL) was run to assess the over-expression of the constructs. Expression of recombinant Myc- β_1 Pix and its mutants was verified by immunoblotting with anti-Myc antibody.

Cdc42 activity assay

Cells were transfected with empty vector, Myc-tagged β_1 Pix alone or in combination with the indicated G α subunit for 24 h. Cells were lysed in lysis/wash buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM MgCl₂, 1 mM EDTA, 1% glycerol, 10 μ g/ml leupeptin, and 10 μ g/ml aprotinin). To measure the active GTP-bound form of Cdc42 GTPase in the cell lysates, we performed a pull-down assay (Upstate) using recombinant glutathione S-transferase (GST)-p21 binding domain (PBD). Aliquots (500 μ g) of the supernatants were incubated with 10 μ g of GST-PBD for 1 h and precipitated by centrifugation. Complexes were boiled in a Laemmli sample buffer and then separated on 15% sodium dodecyl sulfate-polyacrylamide gels. The separated proteins were immunoblotted using a monoclonal anti-Cdc42 antibody.

Results

β_1 Pix interacts with caveolin-1 and G proteins

Several studies have implicated caveolin-1 as a scaffolding protein that enables compartmentalization of specific signaling molecules. ET-Rs have been shown to interact with caveolin-1 and to localize to caveolae [5;6]. Recently, we showed that β_1 Pix binds to ET-Rs [13], therefore we examined whether β_1 Pix interacts with caveolin-1. To evaluate the potential interaction between β_1 Pix and caveolin-1 and the role of β_1 Pix dimerization in this interaction, we expressed wild type β_1 Pix or β_1 Pix dimerization deficient mutant, β_1 Pix Δ (602–611). Using anti-caveolin-1 antibody for immunoprecipitation, we found that wild type β_1 Pix interacts with caveolin-1. This interaction was significantly reduced in cells expressing dimerization deficient β_1 Pix mutant (Fig. 1A), indicating that dimerization is crucial for caveolin-1 binding to β_1 Pix. Caveolin-1 has been shown to interact with different G α subunits through caveolin-1 scaffolding domain [19;20]. Furthermore, this interaction was regulated by the activation state of G proteins and was significantly decreased by GTP γ S [19;21]. Therefore, we set out to determine the effect of G protein activation by AMF on the interaction between β_1 Pix and caveolin-1. Our result showed that GTP γ S induced a strong increase in the interaction between β_1 Pix and caveolin-1 compared to that obtained with GDP, and this interaction was further increased in the presence of AMF (Fig. 1B).

Caveolin-1 is one component of caveolae that functions as scaffolding protein to facilitate signaling between different proteins. G protein α subunits have been identified as caveolin-interacting proteins [19], therefore we were interested to investigate a possible interaction between β_1 Pix and G proteins. Cells were transfected with empty vector or wild type β_1 Pix and lysed in the presence or absence of heterotrimeric G protein activator AMF. We found that β_1 Pix interacts with endogenous G α_q , G α_{12} (Fig. 1C, D) and G α_s (data not shown) and that this interaction was significantly increased in the presence of AMF. Cells transfected with empty vector served as a negative control. To examine whether the interaction between β_1 Pix and

$G\alpha_q$ or $G\alpha_{12}$ depends on the physical integrity of caveolae, cells were treated with methyl- β -cyclodextrin, a cholesterol-depleting agent that disrupts caveolae. Results showed that methyl- β -cyclodextrin blocked the binding of $G\alpha_q$ and $G\alpha_{12}$ to $\beta_1\text{Pix}$ (Fig. 2A, B), but did not block the interaction between $\beta_1\text{Pix}$ and $G\beta_1$ (Fig. 2C).

To determine the role of dimerization on $\beta_1\text{Pix}$ binding to G proteins, cells were transfected with $\beta_1\text{Pix}(\text{wt})$ or $\beta_1\text{Pix}\Delta$ (602–611). Results showed that the binding of $\beta_1\text{Pix}$ to $G\alpha_q$ and $G\alpha_{12}$ is strongly reduced in cells expressing $\beta_1\text{Pix}$ dimerization deficient mutant $\beta_1\text{Pix}\Delta$ (602–611) (Fig. 2D, E).

Constitutively active $G\alpha_q$ and $G\alpha_{12}$ potentiate $\beta_1\text{Pix}$ -induced Cdc42 activation

We have demonstrated that the interaction between $\beta_1\text{Pix}$ and wild type $G\alpha_q$ or $G\alpha_{12}$ is increased in the presence of AMF. In order to investigate the functional significance of these physical interactions, we tested the effect of constitutively active $G\alpha_q$ and $G\alpha_{12}$ subunits on Cdc42 activity. To measure active Cdc42 (Cdc42-GTP), we utilized the pull-down assay that consists of measuring Cdc42-GTP bound to GST-Pak1-PBD in lysates of transfected HMCs. Expression of either $\beta_1\text{Pix}$, $G\alpha_{qQ209L}$ or $G\alpha_{12Q231L}$ alone induced a modest increase in Cdc42 activity (Fig. 3A). The expression of $\beta_1\text{Pix}$ with constitutively active $G\alpha_q$ or $G\alpha_{12}$ induced a significant increase in Cdc42 activity, indicating that the effect of $G\alpha_q$ or $G\alpha_{12}$ and $\beta_1\text{Pix}$ on Cdc42 activity were additive (Fig. 3A).

We have shown that $\beta_1\text{Pix}$ binding to $G\alpha_q$ and $G\alpha_{12}$ requires $\beta_1\text{Pix}$ dimerization. To investigate whether the interaction of $G\alpha_q$ and $G\alpha_{12}$ with $\beta_1\text{Pix}$ is functionally relevant to Cdc42 activation, we analyzed the effect of the expression of $\beta_1\text{Pix}$ dimerization deficient mutant $\beta_1\text{Pix}\Delta$ (602–611), which binding to $G\alpha_q$ and $G\alpha_{12}$ is strongly disrupted, on Cdc42 activity. As shown in Fig. 3B, both $\beta_1\text{Pix}$ and $\beta_1\text{Pix}\Delta$ (602–611) alone were able to increase the amount of active Cdc42. However, the co-expression of $\beta_1\text{Pix}\Delta$ (602–611) and $G\alpha_{qQ209L}$ induced a smaller increase in Cdc42 activity compared to cells transfected with $\beta_1\text{Pix}\Delta$ (602–611) alone. The co-expression of $\beta_1\text{Pix}\Delta$ (602–611) and $G\alpha_{12Q231L}$ induced an activation of Cdc42 that is comparable to that obtained in cells expressing $\beta_1\text{Pix}$ (wt) alone (Fig. 3B). These results indicate that the binding of $G\alpha_q$ and $G\alpha_{12}$ to $\beta_1\text{Pix}$ potentiate Cdc42 activation.

RGS3 inhibits ET-1-induced Cdc42 activation

RGS3 is a member of the regulators of G protein signaling (RGS) family. *In vitro* experiments with purified glutathione S-transferase-fusion RGS3 showed its ability to bind $G\alpha_q$ [22]. We therefore evaluated the ability of RGS3 to modulate $G\alpha_q$ -mediated signaling. We first showed that $\beta_1\text{Pix}$ increased Cdc42 activity, which was further increased by ET-1 stimulation (Fig. 3C). This increase was strongly, although not completely, inhibited in cells expressing RGS3, indicating that ET-1 activation of Cdc42 is mediated at least in part by $G\alpha_q$.

ET-1 increased phospho-Erk1/2 binding to $\beta_1\text{Pix}$

To determine whether $\beta_1\text{Pix}$ is involved in Erk1/2 activation, cells were transfected with empty vector, Myc- $\beta_1\text{Pix}$ or Myc- $\beta_1\text{Pix}\Delta$ (602–611). Cells were stimulated with ET-1 (100 nM for 5 min) and cell lysates were immunoprecipitated with anti-Myc antibody. We found that in unstimulated cells expressing $\beta_1\text{Pix}$ alone phospho-Erk1/2 was bound to $\beta_1\text{Pix}$ and ET-1 stimulation further increased the interaction between phospho-Erk1/2 and $\beta_1\text{Pix}$ (Fig. 4A). Expression of $\beta_1\text{Pix}$ dimerization deficient mutant, $\beta_1\text{Pix}\Delta$ (602–611), significantly decreased ET-1-induced binding of phospho-Erk1/2 to $\beta_1\text{Pix}$ (Fig. 6A). The disruption of caveolae with methyl- β -cyclodextrin strongly inhibited ET-1-induced binding of phospho-Erk1/2 to $\beta_1\text{Pix}$ (Fig. 4B).

ET-1-induced binding of phospho-Erk1/2 to β_1 Pix was strongly inhibited by PKC inhibitor, Ro31-7549 but not by PKA inhibitor, H-89, or pertussis toxin (Fig. 4C). This result further corroborates that $G\alpha_q$ is involved in ET-1-induced activation and binding of Erk1/2 to β_1 Pix, whereas $G\alpha_{i/o}$ and $G\alpha_s$ are not. Altogether, these results suggest that dimerization allows β_1 Pix to bind to caveolin-1 and acts as platform to facilitate the formation of a multi-molecular complex between these proteins which results in Erk1/2 activation and its binding to β_1 Pix.

Discussion

In this study, we have demonstrated that β_1 Pix interacts with caveolin-1, $G\alpha_q$ and $G\alpha_{12}$, and that this interaction was significantly higher in the presence of G protein activator AMF. Co-expression of β_1 Pix and activated mutants of $G\alpha_q$ or $G\alpha_{12}$, induced an additive increase of the amount of active Cdc42. The disruption of β_1 Pix dimerization severely reduced β_1 Pix binding to caveolin-1 and G α subunits and prevented the ability of β_1 Pix to enhance $G\alpha_q$ - and $G\alpha_{12}$ -induced Cdc42 activity. ET-1-induced Cdc42 activation was reduced by RGS3, which block G_q -mediated signaling. ET-1-induced binding of phospho-Erk1/2 to β_1 Pix was inhibited by RGS3 and by PKC inhibitor but not by pertussis toxin or PKA inhibitor, H-89. The disruption of caveolae by methyl- β -cyclodextrin completely blocked the binding of β_1 Pix to $G\alpha_q$ and $G\alpha_{12}$, and reduced interaction between β_1 Pix and phospho-Erk1/2.

Although many members of Rho family small GTPases can be stimulated through G protein-coupled receptors, it remains unclear how the activating signal is transmitted from the receptor to the corresponding GTPase. Active $G\alpha_{13}$ has been shown to directly interact and activate p115RhoGEF [16], while another member of p115RhoGEF, LARG, interacts with $G\alpha_q$ and $G\alpha_{12/13}$ to activate RhoA [15]. These RhoGEFs interact only with active $G\alpha_q$ and $G\alpha_{12/13}$ through their regulator of G protein signaling (RGS) boxes.

We have recently shown for the first time that β_1 Pix, which does not belong to the family of RGS box-containing RhoA-specific GEFs, interacts specifically with $G\alpha_{13}$ in the presence of AMF [13]. Here, we extend the ability of β_1 Pix binding to other G proteins, namely $G\alpha_q$, $G\alpha_{12}$ and $G\alpha_s$ (data not shown). Furthermore, we have previously shown that the activation of $G\alpha_s$ signaling pathway induces the phosphorylation of β_1 Pix by PKA resulting in Cdc42 activation [10]. Here, we show that the co-expression of constitutively active $G\alpha_q$ or $G\alpha_{12}$ and β_1 Pix resulted in an additive increase in Cdc42 activity. $G\alpha_q$ and $G\alpha_{12}$ were unable to enhance Cdc42 activity in the presence of β_1 Pix dimerization deficient mutant indicating that the physical interaction between these $G\alpha_q$ or $G\alpha_{12}$ and β_1 Pix is required for Cdc42 activation. The fact that RGS3 partially blocked Cdc42 activation induced by ET-1, confirms the role of G_q -mediated Cdc42 activation in ET-1 signaling [18] in addition to $G\alpha_{12}$ mediated effect. Therefore, it is reasonable to suggest that the activation of β_1 Pix is induced directly by activated $G\alpha_q$ through stimulation of the ET-Rs.

ET-1-induced binding of activated Erk1/2 to β_1 Pix was partially inhibited by PKC inhibitor and by RGS3, indicating the involvement of $G\alpha_q$, but not that of $G\alpha_{i/o}$ or $G\alpha_s$. This is in agreement with our previous study showing that RGS3 inhibits ET-1-induced Erk1/2 activation [18]. $G\alpha_q$ -mediated activation of PKC can stimulate Erk1/2 through phosphorylation of C-Raf [23]. Erk1/2 can also be stimulated through Cdc42 activation. The disruption of β_1 Pix dimerization or the disruption of caveolae by cholesterol depletion strongly inhibited the binding of $G\alpha_q$ and activated Erk1/2 to β_1 Pix, indicating that this multi-molecular signaling complex requires β_1 Pix dimerization and intact caveolae.

The downstream signaling responses initiated by ET-Rs through $G\alpha_{12/13}$ -dependent mechanisms have been less clearly defined than those dependent on activation of G_q , G_s and G_i proteins. Many GPCRs that are able to activate $G\alpha_q$ also couple to $G\alpha_{12/13}$. In membranes

of aortic smooth muscle cells, ET-Rs have been shown to couple to $G\alpha_q$ as well as $G\alpha_{12/13}$. In our study, we show that in the presence of $\beta_1\text{Pix}$, $G\alpha_{12}$ increases ET-1-induced Cdc42 activation. Therefore, we cannot exclude the possibility that $G\alpha_{12}$ is also involved in ET-1-induced Erk1/2 activation and binding to $\beta_1\text{Pix}$.

Different $G\alpha$ subunits contain caveolin binding motif and directly bind to caveolin-1 through its caveolin scaffolding domain [24]. Our result does not rule out that $\beta_1\text{Pix}$ binds $G\alpha_q$ and $G\alpha_{12}$ through caveolin-1. Interestingly, in this case the role of RGS domain in mediating the binding of $G\alpha$ subunits to p115RhoGEF [16] will be comparable to the role of the scaffolding domain of caveolin-1 in mediating the interaction between $G\alpha$ subunits and $\beta_1\text{Pix}$. Previous studies have indicated that caveolin-1 preferentially interacts with inactive GDP-bound $G\alpha_s$ and inhibits nucleotide exchange [19]. In our case, $\beta_1\text{Pix}$ was able to associate with $G\alpha_s$ (data not shown) and the disruption of caveolae completely blocked the interaction between $\beta_1\text{Pix}$ and $G\alpha$ subunits. Furthermore, activation of heterotrimeric G proteins by AMF showed an increase in the interaction between $\beta_1\text{Pix}$ and caveolin-1, consistent with the increased association found between $\beta_1\text{Pix}$ and AMF-activated $G\alpha_q$ and $G\alpha_{12}$. This strongly suggests that $\beta_1\text{Pix}$ can interact with active $G\alpha$ subunits while still bound to caveolin-1. The results showing that $G\alpha_q$ specifically concentrates in caveolae, whereas $G\alpha_s$ concentrate much more in lipid rafts [20] may explain the sensitivity of inactive $G\alpha_s$ to bind caveolin-1. T_α has been shown to compete with $T_{\beta\gamma}$ to bind caveolin-1 since caveolin binding motif on T_α overlaps with $T_{\beta\gamma}$ binding site [21]. This finding also implies that the trimer (GDP-bound) $T_{\alpha\beta\gamma}$ cannot bind to caveolin-1 since the binding of T_α and $T_{\beta\gamma}$ to caveolin-1 is mutually exclusive. This is in agreement with our finding showing that the binding of $G_{\beta 1}$ to $\beta_1\text{Pix}$ was not blocked by cholesterol depletion. Caveolae have been proposed to provide a microenvironment where different signaling molecules (GPCRs, G proteins, Src family tyrosine kinases) are concentrated. Therefore, localization of ET-Rs to caveolae along with $G\alpha_q$, $\beta_1\text{Pix}$ and Erk1/2 may also contribute to allow efficient and specific signaling by ET-R.

Conclusions

Activation of endothelin receptors leads to the compartmentalization and the binding of $G\alpha_q$ to $\beta_1\text{Pix}$ in caveolae, where dimeric $\beta_1\text{Pix}$ acts as platform to facilitate the binding and the activation of Erk1/2. Our data suggest that the activation of $\beta_1\text{Pix}$ is induced directly by activated $G\alpha_q$, the process triggered by stimulation of endothelin receptors.

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Figure 1.

The binding of caveolin-1 to $\beta_1\text{Pix}$ depends on G protein activation state and $\beta_1\text{Pix}$ dimerization. (A) Cells were transfected with Myc- $\beta_1\text{Pix}$ or $\beta_1\text{Pix}\Delta(602-611)$ for 24 h. Cell lysates were immunoprecipitated with anti-caveolin-1 antibody and the bound proteins were analyzed by Western Blot using anti-Myc antibody. (B) Cells were transfected with Myc- $\beta_1\text{Pix}$ for 24 h and lysed in lysis buffer containing either GDP (10 μM), GTP γ S (10 μM) or AMF (AlCl_3 , 50 μM ; MgCl_2 , 10 mM; NaF, 5 mM). Anti-caveolin-1 was used for immunoprecipitation followed by Western Blot analysis using anti-Myc and anti-caveolin-1 antibodies, respectively. These data are representative of four independent experiments. (C/D) Cells were transfected with empty vector or Myc- $\beta_1\text{Pix}$ and lysed in the absence or presence of AMF. Lysates were immunoprecipitated using either anti- $G\alpha_{q/11}$ (C) or anti- $G\alpha_{12}$ (D) antibodies and the Western Blots were analyzed by anti-Myc antibodies as indicated. Similar results were obtained in three different experiments.

Figure 2.

The binding of $G\alpha_q$ and $G\alpha_{12}$ to β_1 Pix requires caveolae and β_1 Pix homodimerization. Cells were transfected with Myc- β_1 Pix for 24 h and either left untreated or treated with methyl- β -cyclodextrin (5 mM) for 1 h. Cell lysates were immunoprecipitated with anti- $G\alpha_{q/11}$ (A), anti- $G\alpha_{12}$ (B) or anti- $G\beta_1$ (C) antibodies. Precipitated proteins were separated by SDS-PAGE and immunoblotted with anti-Myc, anti- $G\alpha_{q/11}$, anti- $G\alpha_{12}$ or anti- $G\beta_1$ antibodies. The content of $G\alpha$ subunits, $G\beta_1$ and Myc in the total cell lysates indicates the levels of expression of these proteins. These data are representative of similar results obtained in three independent experiments. (D/E) HMCs cells were transfected with Myc- β_1 Pix or Myc- β_1 Pix Δ (602–611) for 24 h. Lysates were immunoprecipitated using anti- $G\alpha_{q/11}$ (D) or anti- $G\alpha_{12}$ (E) antibodies and Western blots were analyzed with anti-Myc antibodies. The expression of Myc- β_1 Pix, $G\alpha_{q/11}$ and $G\alpha_{12}$ was analyzed by Western Blots performed on the total cell lysates. These data are representative of similar results obtained in three independent experiments.

Figure 3.

$G\alpha_q$ and $G\alpha_{12}$ increase β_1 Pix-induced Cdc42 activation. (A) Cells were transfected with empty vector, Myc- β_1 Pix, $G\alpha_{qQ209L}$ or $G\alpha_{12Q231L}$ for 24 h as indicated. (B) Cells were transfected with Myc- β_1 Pix, Myc- β_1 Pix Δ (602–611), $G\alpha_q$ or $G\alpha_{12}$ as indicated. (C) Cells were transfected with β_1 Pix alone or in combination with RGS3 before addition of ET-1 (100 nM) for 5 min. The GTP-bound Cdc42 was measured using pull down assay as described under “Materials and Methods”. The total cell lysates were analyzed for the expression of Myc- β_1 Pix, Cdc42, $G\alpha_q$, $G\alpha_{12}$ or RGS3. Graphs at the bottom of each panel shows fold-increase in Cdc42-GTP densitometric values from three independent experiments normalized to vector which was assigned a value of 1. Results are expressed as mean $\pm$ S.E.M. Data are representative of at least three independent experiments.

Figure 4.

ET-1-induced Erk1/2 activation and binding to β_1 Pix. (A) Cells were transfected with Myc- β_1 Pix or Myc- β_1 Pix Δ (602–611) as indicated for 24 h before addition of ET-1 (100 nM) for 5 min. (B) Cells were transfected with Myc- β_1 Pix and treated with methyl- β -cyclodextrin (5 mM) for 1 h before addition of ET-1. (C) Cells were transfected with Myc- β_1 Pix for 24 h. Cells were incubated with Pertussis Toxin (0.2 μ g/ml) overnight or with H-89 (20 μ M) or Ro31-7549 (20 μ M) for 30 min before stimulation with ET-1 (100 nM) for 5 min. Cell lysates were immunoprecipitated with anti-Myc antibodies and Western blots were analyzed as indicated. These results are representative of three independent experiments.