Novel Mechanisms of G Protein-Dependent Regulation of Endothelial Nitric-Oxide Synthase

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ABSTRACT

Endothelial nitric-oxide synthase (eNOS) plays a crucial role in the regulation of a variety of cardiovascular and pulmonary functions in both normal and pathological conditions. Multiple signaling inputs, including calcium, caveolin-1, phosphorylation by several kinases, and binding to the 90-kDa heat shock protein (Hsp90), regulate eNOS activity. Here, we report a novel mechanism of G protein-dependent regulation of eNOS. We demonstrate that in mammalian cells, the α subunit of heterotrimeric G12 protein (Gα12) can form a complex with eNOS in an activation- and Hsp90-independent manner. Our data show that Gα12 does not affect eNOS-specific activity, but it strongly enhances total eNOS activity by increasing cellular levels of eNOS. Experiments using inhibition of protein or mRNA synthesis show that Gα12 increases the expression of eNOS by increasing half-life of both eNOS protein and eNOS mRNA. Small interfering RNA-mediated depletion of endogenous Gα12 decreases eNOS levels. A quantitative correlation can be detected between the extent of down-regulation of Gα12 and eNOS in endothelial cells after prolonged treatment with thrombin. G protein-dependent increase of eNOS expression represents a novel mechanism by which heterotrimeric G proteins can regulate the activity of downstream signaling molecules.

G12, one of the heterotrimeric guanine nucleotide-binding proteins (G proteins), regulates diverse and complex cellular responses by transducing signals from the cell surface presumably via more than one signaling pathway. The α subunit of G12 (Gα12) regulates the Na+/H+ exchanger activity (Voyno-Yasenetskaya et al., 1994b), extracellular signal-regulated kinase (Voyno-Yasenetskaya et al., 1994b, 1996), and c-Jun NH2-terminal kinase pathways (Prasad et al., 1995; Voyno-Yasenetskaya et al., 1996) and promotes assembly of actin stress fibers (Buhl et al., 1995). It also induces mitogenesis and neoplastic transformation (Xu et al., 1993; Voyno-Yasenetskaya et al., 1994a) and apoptosis (Althoefer et al., 1997; Berestetsky et al., 1998).

It is becoming clear that Gα12 interacts with multiple signaling molecules, which in turn may provide the specificity of Gα12-mediated signaling. The guanine nucleotide exchange factor for RhoA, p115 RhoGEF, was shown to interact with and act as a GTPase activating protein for Gα12 and Gα13 (Kozasa et al., 1998). Another Gα12 (as well as Gα13) interacting protein is cadherin, which is involved in cell-cell adhesion (Meigs et al., 2001). We have previously determined that Gα12 (but not Gα13) binds to αSNAP, a protein involved in membrane trafficking (Andreeva et al., 2005) and Hsp90 (Vaiskunaite et al., 2001), a molecular chaperone that interacts with multiple signal transduction molecules and is essential to a variety of signaling pathways. It is noteworthy that Hsp90 is required for Gα12-induced serum response element activation, cytoskeletal changes, mitogenic response (Vaiskunaite et al., 2001), and probably Gα12 delivery to lipid rafts (Waheed and Jones, 2002).

Endothelial nitric-oxide synthase (eNOS), the isoform that produces endothelium-derived NO, is another important signaling molecule whose activity is regulated by Hsp90 (Garcia-Cardenas et al., 1998). It was demonstrated that Hsp90 is rapidly recruited to the eNOS complex by agonists that stimulate NO production. Moreover, binding of Hsp90 to eNOS enhances activation of the latter, and inhibition of signaling through Hsp90 inhibits agonist-stimulated production of NO (Garcia-Cardenas et al., 1998).

Upon a short exposure of endothelial cells to thrombin,
eNOS activity is increased, without any changes in protein levels. Thrombin causes rapid phosphorylation of eNOS with the maximum effect seen after only 1 min (Thors et al., 2003). However, prolonged incubation with thrombin reduces both activation of eNOS and the protein content (Eto et al., 2001; Ming et al., 2004). Down-regulation of eNOS expression in endothelial cells exposed to thrombin for 24 h can be prevented by an inhibitor of a small GTPase Rhô or by an inhibitor of ROCK (Laufs and Liao, 1998; Ming et al., 2002), a kinase that is a downstream target of Rhô. Activated Rhô seems to down-regulate eNOS expression by decreasing the half-life of eNOS mRNA (Laufs and Liao, 1998).

Gö12 (along with a related Gö13 protein) plays a pivotal role in signal transduction in endothelial cells, in particular in thrombin signaling (Birukova et al., 2004). Involvement of both Gö12 and eNOS in signaling events initiated by thrombin as well as the importance of interaction with Hsp90 for proper functioning of both proteins (Garcia-Cardena et al., 1996; Vaiskunaite et al., 2001) prompted us to investigate whether there might be a functional link between Gö12 and eNOS presumably mediated by Hsp90. Indeed, as reported in this study, we were able to demonstrate that Gö12 can form a complex with eNOS. This interaction, however, did not require Hsp90 and was not dependent on the activation state of Gö12. Furthermore, we found that overexpression of Gö12 led to increased levels of eNOS (and increased total eNOS activity) by a dual mechanism: by increasing the half-life of eNOS protein and of eNOS mRNA. The data from the experiments using siRNA-mediated depletion of endogenous Gö12 and assessment of a quantitative correlation between the extent of thrombin-induced down-regulation of Gö12 and eNOS in endothelial cells are consistent with Gö12 acting to maintain eNOS levels at physiological concentrations of both proteins. These findings suggest that regulation of degradation rate of target proteins and mRNA may represent a novel mechanism by which heterotrimetric G proteins can regulate the activity of downstream signaling molecules.

**Materials and Methods**

**Materials.** Geldanamycin, actinomycin D, and cycloheximide were from Sigma-Aldrich (St. Louis, MO). Polyclonal Gö12 and Gö13 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal HA antibody was purchased from Babco (Richmond, CA). Monoclonal Hsp90 and eNOS antibodies were purchased from BD Transduction Laboratories (Lexington, KY). α-Tubulin monoclonal antibody was from Sigma-Aldrich. Protein A and protein A/G agarose were from Life Technologies, Inc. (Carlsbad, CA), and Santa Cruz Biotechnology, Inc., respectively. Constructs for HA-tagged Gö12 and Gö13 (in pcDNA3) and for p115RhoGEF (in pEXV-Myc) were kindly provided by Silvio Gutkind (Emory Research Institute, Atlanta, GA). Plasmids for EE-tagged Gö12, Gö13, Gö4 and Gö6 (in pcDNA3.1) were from Guthrie Research Institute (Sayre, PA). Plasmids for Gö1 and Gö2 were as described previously (Niu et al., 2003). The cDNA for eNOS was described previously (Garcia-Cardena et al., 1998).

**Cell Culture and Transfection.** Transient transfection of COS-7 cells was performed using LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s instruction. Human umbilical vein endothelial cells (HUVECs) obtained from Clonetics Corporation (Walkersville, MD) were grown in EBM-2 medium supplemented with 10% fetal bovine serum. Cells were cultured on tissue culture dishes coated with 0.1% gelatin. Cells were used between passages 4 and 8.

**Immunoprecipitation and Western Blotting.** eNOS and HA-tagged Gö12 were transiently expressed in COS-7 cells. Cells were lysed in 50 mM HEPES, pH 7.5, 1 mM dithiothreitol, 50 mM NaCl, 5 mM MgCl₂, and 1% Lubrol. In some experiments (see figure legends), NaF and AlCl₃ (final concentrations 5 mM and 50 μM, respectively) were added to the lysis buffer to yield AlF₄⁻. Lysates were normalized for total protein concentration, and proteins were immunoprecipitated with anti-HA antibody and protein A agarose or anti-eNOS antibody and protein A/G agarose for 16 h at 4°C. Immunoprecipitates were washed, precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis in homogenous (8–10%) or gradient (4–25%) gels, transferred onto a nitrocellulose or polyvinylidene difluoride membrane, and probed with appropriate antibodies. Western blots were developed using ECL Plus reagents (Amersham Biosciences Inc., Little Chalfont, Buckinghamshire, UK).

**Subcellular Fractionation of HUVECs.** HUVECs were washed with ice-cold phosphate-buffered saline and then immediately transferred to ice-cold homogenization buffer [50 mM Tris-HCl, pH 7.5, 2.5 mM MgCl₂, 1 mM EDTA, 2.0 mM sodium pyrophosphate, 2 mM NaF and AlCl₃ (final concentrations 5 mM and 50 μM, respectively) were added to the lysis buffer to yield AlF₄⁻]. Lysates were normalized for total protein concentration, and proteins were immunoprecipitated with anti-HA antibody and protein A agarose or anti-eNOS antibody and protein A/G agarose for 16 h at 4°C. Immunoprecipitates were washed, precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis in gradient (4–25%) SDS-polyacrylamide gels.

**NOS Activity Assay.** Nitric oxide synthase activity was measured by monitoring the conversion of [³⁵S]arginine to [³⁵S]citrulline as described previously (Garcia-Cardena et al., 1996). In brief, COS-7 lysates (~75 μl) were incubated in an assay buffer (total volume 100 μl) containing 1 mM NADPH, 5 μM tetrahydrobiopterin, 100 μM calmodulin, 2.5 mM CaCl₂, 10 mM l-arginine, and l-³⁵S]arginine (0.2 μCi; 66 Ci/mmol) for 15 min at 37°C. The reaction was quenched by the addition of 1 ml of ice-cold stop buffer containing 20 mM HEPES, 2 mM EDTA, and 2 mM EGTA, pH 5.5, and the reaction mixture was passed over a 1-ml column containing Dowex A50 WX-8 resin (Na⁺ form, pre-equilibrated in stop buffer). The column was washed with 1 ml of stop buffer, and flow-through was collected directly into scintillation vials. Generated l-³⁵S]citrulline was quantified by scintillation spectrometry.

**siRNA Assay.** Inhibition of Go12 expression was performed using Go12 siGENOME SMARTPool reagent and individual siRNA duplexes (Dharmacon Research, Inc., Lafayette, CO). Assay was performed as described previously (Andreeva et al., 2005).

**Northern Blotting.** Total RNA was isolated from COS-7 cells transiently transfected with the indicated cDNA plasmids using an RNeasy kit from QIAGEN (Valencia, CA). Equal amounts of total RNA (10–20 μg) were separated by 1% formaldehyde-agarose gel electrophoresis, transferred overnight onto Durulon-UV nylon membranes (Stratagene, La Jolla, CA) by capillary action, and the transferred RNA UV cross-linked to the membrane before prehybridization. Radiolabeling of BglIII-XhoI 1.8-kilobase fragment of eNOS was performed using random 9mer primer, [³²P]CTP, and Klenow fragment (Prime-It II random primer kit; Stratagene). The membranes were hybridized with the probes overnight at 50°C in a solution containing 50% formamide, 6× SSC, 5× Denhardt’s solution, 1% SDS, and 100 μg/ml salmon sperm DNA. All Northern blots were subjected to stringent washing conditions (2× SSC, 0.1% SDS at room temperature, followed by 0.1× SSC and 0.1% SDS at 50°C) before autoradiography with intensifying screen at ~80°C for 1 to 24 h.

**Kinetic Analysis.** The cDNAs for eNOS and Gö12Q229L were transiently expressed in COS-7 cells. At 36 h after transfection, the cells were treated with 100 μg/ml cycloheximide or 10 μg/ml actinomycin D for periods of time indicated in the figure legends. Thereafter, cells were collected and protein or mRNA content was analyzed by Western or Northern blotting, respectively.
**Densitometry and Statistical Analysis.** Densitometry of protein bands was performed on scanned images of immunoblots using NIH Image 1.63 (http://rsb.info.nih.gov/nih-image/Default.html). Because the film response may not be linear with enhanced chemiluminescence signal and with the amount of antigen, quantitation was performed from more than one exposure for each experiment to ensure consistency of the results. All densitometric data shown are normalized to internal control (Hsp90 or tubulin, as indicated in figure legends). Quantification of mRNA levels was performed in a similar way using Northern blot autoradiographs. All values are expressed as mean ± S.E. Statistical analysis was performed using Student’s t-test where appropriate. A level of P < 0.05 was considered significant.

**Results**

Gα_{12} and eNOS Form a Protein Complex in Living Cells Independently of the Activation State of Gα_{12}. To test a possibility that Gα_{12} and eNOS might coexist in the same macromolecular complexes, we overexpressed HA-tagged Gα_{12} and untagged eNOS in COS-7 cells and used coimmunoprecipitation with anti-HA antibody, followed by HA-Gα_{12} and eNOS detection by immunoblotting. Indeed, eNOS could be detected in the material immunoprecipitated with HA antibody, both in the absence and in the presence of AlF₄⁻, an activator of Gα subunits that promotes a conformation similar to that of the transition state for GTP hydrolysis (Berman et al., 1996) (Fig. 1A). eNOS was not detectable in HA immunoprecipitates from the cells expressing eNOS but not HA-Gα_{12} (Fig. 1A). These results suggested that Gα_{12} forms a complex with eNOS both in the active and inactive states.

Because HA tag modifies the N terminus of Gα_{12}, it was essential to ensure that HA-tagged Gα_{12} is competent for a transition into its active conformation under our experimental conditions. Gα_{12} interaction with its effector protein p115RhoGEF is known to occur only when Gα_{12} is in the activated state (Vaiskunaite et al., 2001). Therefore, we performed similar coimmunoprecipitation assays with HA-Gα_{12} and p115RhoGEF in the absence or in the presence of AlF₄⁻. HA-Gα_{12} interacted with p115RhoGEF only in the presence of AlF₄⁻ (Fig. 1B), which was consistent with previously published data (Vaiskunaite et al., 2001) and confirmed that the observed independence of the interaction of Gα_{12} with eNOS on the absence or presence of AlF₄⁻ was not an artifact because of the introduction of HA tag. Similar results were obtained with EE-tagged wild-type Gα_{12} and constitutively active EE-Gα₁₂-Q229L (data not shown), which have the primary structure of their N termini intact.

**Interaction of Gα₁₂ and eNOS Does Not Depend on Hsp90.** Because we had initially hypothesized (see Introduction) that Gα₁₂-eNOS interaction might be mediated by Hsp90, we examined whether the disruption of Hsp90 interaction with eNOS and Gα₁₂ would affect Gα₁₂-eNOS interaction.

In the cells transfected with HA-Gα₁₂, antibody against HA specifically immunoprecipitated endogenous Hsp90, whereas in vector-transfected cells, anti-HA antibody did not precipitate Hsp90 (Fig. 2A). These data are consistent with our previously reported results (Vaiskunaite et al., 2001). When cells were additionally transfected with eNOS, immunoprecipitation of HA-Gα₁₂ demonstrated that both Hsp90 and eNOS were present in the complex with Gα₁₂ (Fig. 2A).

To test whether Gα₁₂/Hsp90/eNOS complex formation is indeed mediated by Hsp90, we performed the immunoprecipitation assays in the presence of geldanamycin, an inhibitor that disrupts Hsp90 interactions with target proteins (Pratt, 1998). Cells transfected with HA-tagged Gα₁₂ and eNOS were pretreated with geldanamycin for 1 h, lysed and immunoprecipitated with HA antibody. Our data showed that pretreatment of the cells with geldanamycin disrupted the interaction of Hsp90 with Gα₁₂ (Fig. 2A) as well as with eNOS (Fig. 2B). However, interaction of eNOS with Gα₁₂ was not affected (Fig. 2A). Together, these data suggest that association of Gα₁₂ with eNOS is not mediated by Hsp90.

**Overexpression of Gα₁₂ Increases eNOS Levels and Total eNOS Activity.** While performing the immunoprecipitation experiments described above, we noticed that expression levels of eNOS tended to be somewhat higher in cells that were cotransfected with Gα₁₂ compared with eNOS alone, suggesting that coexpression with Gα₁₂ might upregulated Gα₁₂ and eNOS form a protein complex in vivo independently of the activation state of Gα₁₂. A, activation-independent binding of Gα₁₂ to eNOS. COS-7 cells (60-mm dish) were transiently transfected with 1 μg of plasmids encoding HA-tagged Gα₁₂ and untagged eNOS, or empty vector (pcDNA3) as indicated. Forty-eight hours after transfection, cells were lysed and immunoprecipitated (IP) with anti-HA antibody in the absence or presence of AlF₄⁻. Immunoprecipitates and total cell lysates were analyzed by Western blotting (WB) with anti-HA and anti-eNOS antibodies, respectively. B, activation-dependent binding of Gα₁₂ to p115RhoGEF. COS-7 cells were transfected and analyzed as described in A, except that a construct encoding Myc-tagged p115RhoGEF and anti-Myc antibody were used instead of eNOS construct and anti-eNOS antibody. Similar results were obtained in three independent experiments.
regulate eNOS. Separate experiments with varying ratios of eNOS and Gα12 determined that the effect of Gα12 on eNOS levels increased at lower eNOS-to-Gα12 ratios (Fig. 3, compare 50 and 450 ng of transfected Gα12). The effects of wild-type Gα12 and of mutationally activated Gα12Q229L were similar (Fig. 3), showing that Gα12 increases eNOS levels independently of its activation state. This is in line with the observed similar ability of Gα12 to form a complex with eNOS in the absence and in the presence of AlF$_4^{-}$ (Fig. 1).

To assess how Gα12 would affect the activity of eNOS, we transfected COS-7 cells with Gα12 or vector in the presence of increasing amounts of eNOS. eNOS activity, quantified by analyzing the conversion [3H]arginine to [3H]citrulline in the presence of overexpressed Gα12, was considerably higher in the cells transfected with Gα12 and eNOS than in the cells transfected with the same amounts of eNOS construct without Gα12 (Fig. 4, compare right and left). Gα12 alone did not have a detectable effect on endogenous NOS activity (Fig. 4). Analysis of the same cell lysates by immunoblotting confirmed that eNOS levels in the cells cotransfected with Gα12 were considerably higher than in the cells transfected with eNOS alone (Fig. 4), suggesting that the increase in total eNOS activity in the presence of overexpressed Gα12 may be because of the increased levels of eNOS protein, rather than to its increased specific activity. Indeed, normalization of total eNOS activity to the amounts of eNOS in the cell extracts and comparison of eNOS activity in COS-7 cells expressing similar levels of eNOS in the absence and in the presence of coexpressed Gα12 did not show a significant correlation between the presence of Gα12 and specific activity of eNOS (data not shown). Thus, the experiments described above indicated that coexpression with Gα12 increases levels of eNOS protein and therefore total eNOS activity, but it does not affect specific activity of eNOS.

In addition, it should be noted that increasing the levels of eNOS in COS-7 cells was accompanied by a progressive decline in its specific activity (Fig. 4, inset). These observations are in line with those reported for cultured bovine aortic endothelial cells where hypoxia increased eNOS expression with concomitant decrease in eNOS-specific activity, resulting in an unchanged total NO production (Arneth et al., 1996).

The above-mentioned observations raised a question of whether these properties are unique to Gα12, or whether other heterotrimeric G proteins are also able to affect eNOS expression levels. Although detailed comparison of different G proteins in this respect is beyond the scope of this work, our preliminary data suggest that Gα12 is as potent in both affecting eNOS levels and the ability to interact with eNOS as Gα12Q229L, whereas no effect of Gα12 and Gαz as well as Gβγ could be detected (data not shown). Overexpression of Gα12 seemed to increase eNOS levels; this increase, however, was reversed by a further elevation of Gα12 levels, suggesting possible counteraction of more than one mechanism (data not shown).

$\text{Gα12 Stabilizes Both eNOS Protein and eNOS mRNA.}$ We next addressed a possible mechanism how Gα12 affects the levels of eNOS. Because eNOS expression in COS-7 cells was driven not by endogenous eNOS promoter, but by a constitutively active eNOS-unrelated cytomegalovirus promoter, regulation at transcriptional level was unlikely. To rule out a possibility of transcriptional regulation, we analyzed the sequence of eNOS cDNA used in this study and determined that only a short stretch of its 5’-untranslated region, which lacked the eNOS promoter region, was present in the plasmid. In addition, we have previously shown on a number of occasions that Gα12 does not induce the cytomegalovirus promoter (Voyno-Yasenetskaya et al., 1996; Berestetskaya et al., 1998; Niu et al., 2001; Vaikunnaite et al., 2001). These considerations allowed us to exclude that Gα12 might transcriptionally regulate expression of eNOS. Therefore, we examined whether Gα12 might affect stability of eNOS protein and/or mRNA.

We used a kinetic analysis of eNOS and Gα12 expression in the presence of cycloheximide to suppress protein synthesis (Fig. 5). Total cell lysates were analyzed by Western blotting (Fig. 5A). Relative eNOS expression was calculated as the ratio between signal intensities of eNOS and Gα12 bands and...
normalized to that in the cells before cycloheximide addition (Fig. 5B). Cycloheximide decreased the levels of both eNOS and endogenous Gα12 in a time-dependent manner (Fig. 5), although the decrease in the levels of Gα12 was much slower and almost within the experimental error (Fig. 5C). After 9 h of cycloheximide treatment, relative eNOS levels decreased by 40 to 70% (eNOS half-life 6.0 ± 1.3 h; n = 3). In contrast, in the presence of Gα12, no statistically significant decline in relative eNOS levels was observed (Fig. 5B). Similar results were obtained when constitutively active Gα12Q229L was used (data not shown). The expression of Hsp90 was not changed after 9 h of protein synthesis inhibition (Fig. 5A). These data indicate that Gα12 is able to stabilize eNOS protein.

To determine whether stabilization of eNOS mRNA might also contribute to Gα12-dependent increase in eNOS protein levels, we used a kinetic analysis in the presence of a transcription inhibitor actinomycin D. COS-7 cells expressing eNOS alone or eNOS and Gα12Q229L were pretreated with actinomycin for 5, 10, and 25 h, or not treated. Thereafter, Northern blot analysis of total RNA was performed and the eNOS band intensity relative to the 28S ribosomal RNA was assessed whether a decrease in endogenous Gα12 would affect eNOS levels. We used siRNA-mediated Gα12 depletion, which decreased endogenous Gα12 content by 40 to 60% both in COS-7 cells and in HUVECs (Fig. 7). Gα12 depletion was associated with a considerable decrease in eNOS levels, both when it was expressed in COS-7 cells, and, most importantly, endogenous eNOS in HUVECs (Fig. 7). These data indicate that the stabilizing effect of Gα12 on eNOS levels does take place at physiological concentrations of both proteins.

**Thrombin Decreases Expression of Gα12 and eNOS in Endothelial Cells.** Prolonged treatment of HUVECs with thrombin was shown to decrease eNOS expression (Eto et al., 2001; Ming et al., 2002; Ming et al., 2004). This phenomenon was reproducible in our experiments. Moreover, we found that prolonged treatment with thrombin also decreased the levels of Gα12 (Fig. 8). When HUVECs were lysed by mild sonication in the absence of detergent and then separated by centrifugation into three particulate fractions and a soluble fraction (see Fig. 8 legend and Materials and Methods for details), eNOS could be detected exclusively and Gα12 mainly in the particulate fractions (data not shown). To
examine whether there is a quantitative correlation between the extent of down-regulation of eNOS and Gar12, we produced a reciprocal plot of the levels of the two proteins in different fractions after 24-h thrombin treatment, normalized to their levels in respective fractions in the cells not treated with thrombin. Plotting the data from subconfluent and confluent HUVEC cultures showed a clear quantitative correlation (correlation coefficient 0.93) between the extent of down-regulation of Gar12 and that of eNOS (Fig. 8). Although these data cannot rule out a common down-regulation mechanism acting independently on Gar12 and on eNOS, these observations are also compatible with Gar12 acting upstream of eNOS to regulate its cellular levels.

Discussion

It is becoming clear that intracellular signaling events can be regulated by heterotrimeric G proteins not only via second messengers such as cyclic AMP but also via direct interactions involving Gar or Gβγ subunits and other signaling proteins. Several important signaling molecules have been shown to interact with Gar12, including several RhoGEFs (Kozasa et al., 1998; Fukuhara et al., 1999, 2000) that act between the actin cytoskeleton and the plasma membrane and regulate organization of cortical actin (Vaiskunaite et al., 2000); cadherin, a protein that mediates cell-cell interactions, and upon Gα12 binding, releases a transcriptional activator β-catenin (Meigs et al., 2001); αSNAP, a protein involved in membrane trafficking that in complex with Gar12, increases cadherin presence at endothelial junctions (Andreeva et al., 2005); and zonula occludens proteins 1 and 2 that probably regulate properties of the tight junctions (Meyer et al., 2002). It was also shown that Gar12 interacts with a molecular chaperone Hsp90 and that this interaction is required for Gar12 function (Vaiskunaite et al., 2001), possibly via Hsp90-dependent targeting of Gar12 to lipid rafts (Jones and Gutfink, 1998).

Because, on one hand, Hsp90 is also an important functional partner of eNOS (Garcia-Cardena et al., 1998; Martinez-Ruiz et al., 2005) and eNOS levels are regulated by prolonged thrombin treatment (Eto et al., 2001); and on the other hand, Gar12 is an essential component in thrombin signaling, we initially hypothesized that there might be a functional link between Gar12 and eNOS, mediated by Hsp90. Although such a role of Hsp90 could not be confirmed in the course of our study, this initial hypothesis led us to a finding that Gar12 and eNOS do interact in living cells when overexpressed using the COS-7 cell model. Although a traditional general paradigm for Gar proteins has been that they transmit signals to their targets while in the GTP-bound (i.e., activated) state, the Gar12-eNOS interaction was found to occur independently of the activation state of Gar12. Similar observations have been reported for Gar12 interaction with Hsp90 (Niu et al., 2001), PP2A (Zhu et al., 2004), and αSNAP (Andreeva et al., 2005).

The functional consequences of the Gar12-eNOS interaction are also “noncanonical” in terms of a typical G protein-mediated regulation of its effector: Gar12 does not seem to affect specific activity of eNOS, but increases the cellular levels of eNOS. An intriguing finding of this work is that this increase in eNOS expression occurs via two probably distinct mechanisms, resulting in an increase in half-life of both eNOS mRNA and eNOS protein. To the best of our knowledge, this

Fig. 7. Depletion of endogenous Gar12 by siRNA leads to eNOS down-regulation. HUVECs and COS-7 cells (24-well plate) were transfected with Gar12 siRNA duplexes or control siRNA as indicated and eNOS (COS-7 only; 20 ng/well). Forty-eight hours after transfection, levels of eNOS and Gar12 were determined by WB (left) and quantitated by densitometry (right). Data shown are means, and error bars are S.E. (n = 3 for HUVECs; n = 4 for COS-7). White columns, Gar12, gray columns, eNOS.

Fig. 8. Correlation between thrombin-induced down-regulation of Gar12 and eNOS in HUVECs. Confluent or subconfluent HUVEC cultures were treated with thrombin (50 nM) for 24 h. Cells were lysed by sonication and fractionated by centrifugation as described under Materials and Methods. Levels of endogenous Gar12 and eNOS as well as tubulin were assessed by WB using respective antibodies. Left, representative blots of P1 fractions from confluent or subconfluent HUVECs as indicated. Right, correlation between the extent of down-regulation of Gar12 and eNOS in particular fractions of the two HUVEC cultures (no eNOS could be detected in P100 fractions of either subconfluent or confluent HUVECs). Values were obtained by densitometry of scanned images and normalized to tubulin. Correlation coefficient calculated for this data set is 0.93. The experiment was repeated twice with similar results.
is the first demonstration of the ability of a heterotrimeric G protein to regulate the activity of downstream signaling molecules by affecting their degradation rate.

Our findings raise a question of whether other heterotrimeric G proteins might possess similar properties toward eNOS as Gα12. Although a systematic exploration of this issue remains to be carried out, our preliminary work suggests that these properties might be a characteristic feature of the Gα12/13 subfamily, although we could also detect some effects of Gα on eNOS expression.

Recent work provided evidence that activation of Rho and its effector ROCK results in inhibition of eNOS expression, probably via destabilization of eNOS mRNA (Eto et al., 2001). Although the precise mechanisms how Gα12 stabilizes both eNOS mRNA and protein remain to be elucidated, it is tempting to speculate that Gα12 could act as an mRNA-stabilizing protein that stabilizes eNOS mRNA. Regulation of eNOS mRNA by RNA-binding proteins has been previously documented. Monomeric actin has been found to be a predominant component of a ribonucleoprotein that binds to the 3′-untranslated region of eNOS mRNA (Searles et al., 2004).

Because Gα12 is a Rho activator (Kozasa et al., 1998; Fukuhara et al., 1999, 2000), it would be predicted to exert opposite effects on eNOS expression: to destabilize eNOS via activation of Rho and ROCK (Eto et al., 2001) and to stabilize it by increasing half-lives of eNOS mRNA and protein as described in this work. Therefore, it is essential to establish whether all these effects take place in vivo. In this respect, important observations reported here are that siRNA-mediated down-regulation of endogenous Gα12 in HUVECs leads to decreased levels of eNOS and that there is a robust quantitative correlation between the extent of down-regulation of Gα12 and eNOS in untransfected HUVECs after prolonged thrombin treatment. These results suggest that Gα12 does have a stabilizing effect on eNOS at physiological concentrations of both proteins, at least in cultured HUVECs.

Although probable destabilizing effect of Gα12 on eNOS via Rho-ROCK and the stabilizing effects of Gα12 reported here seem to be counteractive at first glance, they may actually complement each other, taking into account their physiological context and timing. Indeed, in unstimulated cells, steady levels of eNOS would be maintained by a balance of various mechanisms, including stabilizing effects of Gα12 reported in this study. Rho-ROCK activation by thrombin would lead to eNOS down-regulation as reported in Eto et al. (2001). At the same time, thrombin would induce down-regulation of Gα12 by as yet undefined mechanism, which would reduce the stabilizing effect of Gα12 on eNOS and further down-regulate it. On the other hand, Gα12 down-regulation could be downstream of Rho-ROCK activation and thus mediate the Rho-ROCK effect.

In conclusion, we have characterized a novel signaling module of Gα12 and eNOS and described a novel functional link between these proteins. G protein-dependent stabilization of a target protein reported here represents a novel mechanism by which heterotrimeric G proteins can regulate the activity of downstream signaling molecules.

References


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