The Proline-rich Domain of Dynamin-2 Is Responsible for Dynamin-dependent in Vitro Potentiation of Endothelial Nitric-oxide Synthase Activity via Selective Effects on Reductase Domain Function

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The GTPase dynamin-2 (dyn-2) binds and positively regulates the nitric oxide-generating enzyme, endothelial nitric-oxide synthase (eNOS) (Cao, S., Yao, Y., McCabe, T., Yao, Q., Katusic, Z., Sessa, W., and Shah, V. (2001) J. Biol. Chem. 276, 14249–14256). Here we demonstrate, using purified proteins, that this occurs through a selective influence of the dyn-2 proline-rich domain (dyn-2 PRD) on the eNOS reductase domain. In vitro studies demonstrate that dyn-2 PRD fused with glutathione S-transferase (GST) binds recombinant eNOS protein specifically and with binding kinetics comparable with that observed between dyn-2 full-length and eNOS. Additionally, GST-dyn-2 PRD binds the in vitro transcribed 35S-eNOS reductase domain but not the 35S-eNOS oxygenase domain. Furthermore GST-dyn-2 PRD binds a 35S-labeled eNOS reductase domain fragment (amino acids 645–850) that partially overlaps with the FAD binding domain of eNOS. A recombinant form of the SH3-containing protein Fyn competes the binding of recombinant eNOS protein with dyn-2 PRD, thereby implicating the SH3-like region contained within this reductase domain fragment as the dyn-2 binding region. Mammalian two-hybrid screen corroborates these interactions in cells as well. Functional studies demonstrate that dyn-2 PRD selectively potentiates eNOS activity in a concentration-dependent manner in an order of magnitude similar to that observed with dyn-2 full-length and in a manner that requires calmodulin. A full-length dyn-2 PRD does not influence eNOS oxygenase domain function or ferricyanide reduction, it does potentiate the ability of recombinant eNOS to reduce cytochrome c, supporting an influence of dyn-2 PRD on electron transfer between FAD and FMN. (These data indicate that the binding domains of dyn-2 and eNOS reside within the dyn-2 PRD domain and the FAD binding region of the eNOS reductase domains, respectively, and that dyn-2 PRD is sufficient to mediate dyn-2-dependent potentiation of eNOS activity, at least in part, by potentiating electron transfer.)

Endothelial nitric-oxide synthase (eNOS) is a membrane-associated protein that catalyzes the conversion of L-arginine to L-citrulline and nitric oxide (NO) (1). eNOS function is regulated in part by post-translational mechanisms including acylation, phosphorylation, and protein interactions (2–7). Indeed, specific proteins have been identified which interact with eNOS, thereby regulating enzyme function, including the large GTPase, dynamin-2 (dyn-2) (8–12). With regard to the latter, it has been demonstrated that dyn-2 specifically binds eNOS in a direct manner and potentiates the ability of eNOS to convert L-arginine to L-citrulline in a concentration-dependent manner (12). However, delineation of binding domains and the mechanism of activation remain unexplored.

eNOS is a bi-domain enzyme that requires a number of cofactors and substrates to generate NO optimally via an orchestrated electron transfer mechanism (1). An oxygenase domain (amino acids 1–491) contains binding sites for tetrahydrobiopterin, heme, and L-arginine, and a reductase domain (amino acids 492–1205) contains binding sites for calmodulin, FMN, FAD, and NADPH (1). Electron flux is initiated at the reductase domain where NADPH-derived electrons are transferred sequentially through the bound flavins, FAD and FMN. Electron transfer from the reductase domain to the oxygenase domain is facilitated by calmodulin and allows for reduction of heme iron and the ensuing binding and activation of oxygen. Subsequent oxidation of the amino group of L-arginine allows for the formation of L-citrulline, water, and NO (1). Assessment of the individual reductase and oxygenase components of this biochemical paradigm allows for mechanistic dissection of how eNOS is influenced by putative regulatory events. Specifically, eNOS reductase domain function can be dissected in isolation from the oxygenase domain by exploiting the ability of moieties within this domain to transfer electrons to exogenous heme protein acceptors (13–16). Specifically, the rate of electron transfer from the eNOS electron acceptor ferricyanide (FeCN), which accepts electrons directly from the FAD; the rate of electron transfer from FAD to FMN can be estimated by utilizing the exogenous electron acceptor cyanochrome c (15, 17). Conversely, oxygenase domain function can be analyzed, independent of reductase domain-dependent heme reduction, by measuring NO synthesis from the

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§ The abbreviations used are: eNOS, endothelial nitric-oxide synthase; CAT, chloramphenicol acetyltransferase; dyn-2, dynamin-2; EPSPS, 4-(2-hydroxyethyl)-1-piperazinopropanesulfonic acid; FeCN, ferricyanide; GED, GTPase effector domain; GST, glutathione S-transferase; HEK, human embryonic kidney; l-NAME, l-nitroarginine methyl-ester; NO, nitric oxide; NOHA, Nα-hydroxy-L-arginine; PHD, pleckstrin homology domain; PRD, proline-rich domain; SH3, Src homology domain.
NOS enzyme reaction intermediate N^ω-hydroxy-L-arginine (NOHA) (14, 18, 19).

Members of the dynamin family of proteins, including dyn-2, are recognized as modulators of membrane scission events (20). However, these proteins also facilitate specific and well-characterized signaling functions by virtue of distinct protein interactions (21). In this regard, the dyn-2 protein consists of several characterized subdomains: the GTPase domain (amino acids 1–399), pleckstrin homology domain (PHD; amino acids 521–623), GTPase effector domain (GED; amino acids 623–746), and the proline-rich domain (PRD; amino acids 746–870). Each of these domains maintains distinct functions, and they act in concert to facilitate the cellular functions of dyn-2 (22). The GTPase domain is responsible for the binding and hydrolysis of GTP, events that are promoted by GED. PHD and PRD regions are prominently implicated in regulatory binding interactions. More specifically, PHD binds phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-phosphate, whereas PRD binds specific proteins, often notable for the presence of an SH3 domain, including phospholipase Cγ, Nck, AP-2, Grb2, and phosphatidylinositol 3-kinase (23–30).

Here we seek to elucidate further interaction regulation mechanisms of dyn-2 and eNOS, using recombinant proteins. The present study demonstrates that dyn-2 binds eNOS by virtue of dyn-2 PRD and conversely that the binding domain of dyn-2 on eNOS is contained within its reductase domain, specifically within a sequence overlapping the FAD binding domain. This interaction is competed by the SH3-containing protein Fyn, thereby implicating the SH3-like region within this eNOS reductase domain fragment as the dyn-2 PRD binding site. Furthermore, glutathione S-transferase (GST)-dyn-2 PRD potentiates eNOS activity in a concentration-dependent manner, with potency similar to that of the full-length protein and in a manner that requires bound calmodulin. Analysis of individual NOS subdomain function demonstrates that although GST-dyn-2 PRD does not influence eNOS oxygenase domain reductase activity independently, it does influence eNOS reductase domain activity by potentiating electron transfer between the reductase domain flavins, FAD and FMN. Thus these studies provide mechanistic insights as to how dyn-2 enhances eNOS protein function in vitro.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Proteins—Recombinant eNOS protein was purified from Escherichia coli as we and others have described previously (12, 31). In brief, bovine eNOS in the plasmid pCW was coexpressed with pGroEL5 plasmid into protease-deficient E. coli. eNOS was purified from extracts using a 2',5'-purified recombinant eNOS (0–15 pmol) with GST-dyn-2 subdomain proteins with purified recombinant eNOS from E. coli was performed by incubating recombinant eNOS protein (15 pmol) with GST-dyn-2 fusion proteins (15 pmol), or GST beads alone, at 4 °C overnight, in a 300-µl reaction buffer containing 50 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100, pH 7.5. For experiments with purified recombinant eNOS (3 pmol) was incubated with varying amounts of recombinant Fyn (0–15 pmol) and with GST beads that contained 10 pmol of GST-dyn-2 PRD, or alternatively GST beads alone, overnight at 4 °C in 300 µl of binding buffer. Bound proteins were washed three times with a buffer containing 50 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, then eluted with Laemmli buffer and used for gel electrophoresis. Bound proteins were analyzed by SDS-PAGE and Western blot analysis, using an eNOS monoclonal antibody and a Fyn monoclonal antibody (Transduction Laboratories, Lexington, KY) (33). In vitro binding of GST-fused dyn-2 subdomain proteins with in vitro transcribed 35S-eNOS subdomains and reductase domain fragments was carried out by incubating 15 pmol of GST-dyn-2 PRD beads alone with a fixed concentration of in vitro translated eNOS (3 µl of rabbit reticulocyte lysate) in 300 µl, using incubation and wash conditions identical to those described above. Bound 35S-eNOS was examined by SDS-PAGE and autoradiography of dried gels. Calculation of the equilibrium dissociation constant (Kd) was performed by incubating a fixed concentration of GST-dyn-2 or GST-dyn-2 PRD beads (5 nM) with purified recombinant eNOS (0–640 nm), premixed with proportionate volume of 35S-eNOS (0–32 µl) used as a radiolabel tracer in a 300-µl reaction mix. Previous analyses performed over a logarithmic range of immobilized GST-dyn-2 protein concentrations (5–45 nm) incubated with saturating concentrations of eNOS ligand have demonstrated that varying immobilized GST-dyn-2 protein concentrations in in vitro binding experiments do not affect the Kd estimation (12). Quantitation of bound and free 35S-eNOS, assessed directly by scintillation counting, allowed for determination of bound and free recombinant eNOS. Radioactive counts detected after incubation of GST beads with 35S-eNOS, which were of low level, were attributed to background and were subtracted from all subsequent values. Kd was calculated by Scatchard plot analysis of bound and free levels of recombinant eNOS.

Mammalian Two-hybrid Screen—A GALA-based mammalian two-hybrid screen was used to detect subdomain interactions in HEK cells (34). All tissue culture reagents for HEK cells were obtained from Invitrogen. pM and pVP16 vectors were used to create fusion proteins containing the GAL4 DNA binding domain and DNA activation domain, respectively (35) (mammalian two-hybrid matchmaker assay, Clontech). CDNA encoding eNOS full-length and subdomains and dyn-2 full-length and subdomains were subcloned into vectors pM and pVP16, respectively, using PCR with primers incorporating restriction endonuclease cutting sites for BanHI and XbaI, and HindIII and XhoI, respectively. CDNA encoding reductase domain fragments with amino acids 511–850, 511–645, and 645–850 were generated by PCR with primers incorporating restriction sites for EcoRI and BanHI and inserted into pM. HEK cells, grown in 12-well plates, were transfected with pM and pVP16 vectors (0.2 µg) containing appropriate inserts, as well as vectors encoding CAT and Renilla luciferase reporter vectors (0.1 µg and 0.01 µg, respectively). Cell lysates were prepared 24 h following transfection. CAT reporter gene expression was quantified from cell lysates from triplicate wells using a spectrophotometric assay (CAT enzyme-linked immunosorbent assay, Roche Molecular Biochemicals). Variation in transfection efficiency and protein concentration was corrected by normalizing CAT readouts with Renilla luciferase values. Negative controls used each experiment included transfection with pM-53, which encodes mouse p53 protein, and pVP16-T, which encodes SV40 large T-antigen, which is known to interact with p53.

In Vitro Activity Assays—NOS activity from E. coli-derived recombinant eNOS protein was assessed by measuring the L-NAME-inhibited conversion of 3H-labeled L-arginine to 3H-L-NAME. Assays of in vitro activity were performed in reaction mix containing vectors encoding bovine full-length, subdomains, or reductase domain fragments of eNOS DNA (or alternatively negative control containing no DNA), T7 RNA polymerase, and [35S]methionine, was incubated at 30 °C for 90 min. Translation products were examined by SDS-PAGE analysis and autoradiography of dried gels. Purity and specificity of these reagents are shown in the autoradiograph in Fig. 2, B and D. Recombinant Fyn was obtained from PanVera (Madison, WI).
was incubated with 0.25 pmol of purified recombinant eNOS protein for 60 min at 4 °C in 300 μl of binding buffer identical to that described for in vitro binding assays. In some experiments the concentration of dyn-2 PRD protein was varied between 1.5 and 8 pmol to generate a molar range of 0.25:1, to 1:2.5:1, of dyn-2 PRD to eNOS. To determine NOS activity, duplicate samples of the preincubated recombinant proteins were added to a 50-μl reaction mixture containing 1 μM NADPH, 3 μM tetrahydrobiopterin, 100 nM calmodulin, 2.5 μM CaCl₂, 10 μM l-arginine and l-[3H]arginine (0.2 μCi) at 37 °C for 20 min. In some experiments the calmodulin concentration was varied from 0 to 100 nM. All samples were analyzed in the presence and absence of the NOS inhibitor L-NAME (1 mM) for specificity of effect. The reaction mix was terminated by the addition of 1 ml of cold stop buffer (20 mM HEPES, 2 mM EDTA, 2 mM EGTA, pH 5.5) and passed over a Dowex AG 50W-X8 resin column. Radiolabeled counts/min of generated l-citrulline were measured and used to determine L-NAME-inhibitable NOS activity.

Cytochrome c Reduction Assay and FeCN Reduction Assay—Components of reductase domain function were determined by measuring the ability of E. coli-derived recombinant eNOS to reduce cytochrome c and alternatively FeCN, in an NADPH-dependent manner using spectrophotometric assays (13–17, 36). GST, GST-dyn-2, and GST-dyn-2 subdomains were eluted with reduced glutathione and dialyzed. 4 nM recombinant eNOS protein was incubated for 1 h with 4 nM GST-fused dyn-2 subdomain protein in 300 μl of reaction buffer buffer containing 50 mM HEPES, pH 7.4, 250 mM NaCl at 4 °C. In some experiments, assays were performed using varying concentrations of GST-dyn-2 PRD (2–7 nM) or alternatively, preboiled GST-dyn-2 PRD protein. For cytochrome c reduction assay, 100-μl aliquots from the preincubation mix were added to a 1-ml reaction containing 50 mM HEPES, pH 7.4, 250 mM NaCl, 0.2 mM NADPH, 0.12 μM cytochrome c, 0.1 mM NADP, 0.12 μM calmodulin, and 0.2 mM CaCl₂. Cytochrome c reduction was measured at 550 nm at 25 °C, using a Beckman spectrophotometer with temperature control (model DU 650), with slight modification from the protocol described by McCabe et al. (13). Briefly, the reaction was monitored for 60 s, immediately upon addition of the preincubated recombinant protein mix to the reaction buffer, a period during which the reduction rate was linear. The change in absorbance was calculated between the 30-s period of 0–40 s. Turnover number was calculated using the absorbance change during this 30-s interval and an extinction coefficient of 0.021 μM⁻¹. For FeCN reduction assay, 100-μl aliquots from the preincubation mix were added to a 1-ml reaction buffer containing 50 mM HEPES, pH 7.6, 250 mM NaCl, 0.2 mM NADPH, 0.12 μM cytochrome c, 0.2 mM CaCl₂. Reaction was initiated with the addition of 0.5 mM potassium ferricyanide, after which the reaction was monitored for 10 min at 420 nm at 25 °C. The turnover number was then calculated using an extinction coefficient of 1.02 μM⁻¹.

NADPH-independent Oxygenase Domain Assay—Selective, NADPH-independent function of the oxygenase domain was assessed by determining the ability of E. coli-derived recombinant eNOS to convert NOHA into nitrite, after which the reaction was monitored for 10 min at 420 nm at 25 °C. The turnover number was then calculated using an extinction coefficient of 1.02 μM⁻¹.

RESULTS

Dyn-2 PRD and eNOS

Fig. 1. GST-dyn-2 binds eNOS by virtue of the dyn-2 PRD. Binding assays were performed by incubating E. coli-derived purified recombinant eNOS with GST-dyn-2 subdomain fusion proteins. Bound proteins were assessed by gel electrophoresis and Western blot analysis. A, Coomassie-stained SDS-PAGE demonstrates the purity of GST-dyn subdomains (GTPase, PHD, GED, and PRD), as evidenced by a single dominant band of the correct molecular size for each subdomain. B, the representative Western blot demonstrates that when incubated at an equimolar ratio, GST-dyn-2 PRD binds recombinant eNOS protein. Conversely, other GST-dyn-2 subdomains do not bind eNOS. The blot is representative of three independent experiments that yielded similar results. C, a series of concentrations (0–640 nM) of E. coli-derived purified recombinant eNOS protein, premixed with proportionate amounts of [35S]-eNOS tracer (0–32 μl), was incubated with 5.0 nM GST-dyn-2 or alternatively GST-dyn-2 PRD. Bound [35S]-eNOS and an [35S]-eNOS standard curve were analyzed directly by scintillation counting. Scatchard plot analysis of bound and free recombinant eNOS is shown with individual data points from a representative experiment using GST-dyn-2 (black triangles; K_d = 62.4 ± 16.7 nM, B_max = 0.97 ± 0.12 nM) and GST-dyn-2 PRD (white squares; K_d = 129.8 ± 12.6, B_max = 1.13 ± 0.16). K_d and B_max, the maximal binding of eNOS ligand at the indicated concentrations of recombinant proteins, are mean values derived from three independent experiments, each with duplicate readings.

from E. coli, incubated at a 1:1 molar ratio with the characterized subdomains of dyn-2, including GTPase, PHD, GED, and PRD, each in the form of a GST fusion. The Coomassie stained SDS-polyacrylamide gel, in Fig. 1A, depicts the purity of the GST-dyn-2 subdomain proteins as shown by a single dominant protein band of the corresponding molecular size. As seen in the representative Western blot in Fig. 1B, the specific binding of purified recombinant eNOS is detected with GST-dyn-2 PRD, when both proteins are incubated at an equimolar ratio. Conversely, no binding is detected between recombinant eNOS and other GST-dyn-2 subdomains.

We next sought to compare the binding affinity between eNOS and dyn-2 PRD in vitro using radiolabel tracer experi-
ments and Scatchard analysis of binding data. 5.0 nM GST-dyn-2 was incubated with a logarithmic range of concentrations of purified recombinant eNOS protein (0–640 nM) premixed with proportional volumes of $^{35}$S-eNOS tracer. Bound and free radioactive counts were measured directly by scintillation counting in duplicate. In Fig. 1C, a Scatchard plot analysis of binding data is shown. Scatchard analysis demonstrates a $K_d$ of 62.4 nM between eNOS and dyn-2 (black triangles), similar to that reported previously (12). $K_d$ analysis of eNOS and dyn-2 PRD (white squares) was detected to be 129.8, which is comparable although somewhat higher than that observed with the dyn-2 full-length. $B_{max}$, the maximal binding of eNOS ligand at the indicated concentrations of recombinant GST proteins, was similar, 0.97 ± 0.12 nm and 1.13 ± 0.16 nm for GST-dyn-2 and GST-dyn-2 PRD, respectively. The somewhat greater affinity of dyn-2 full-length for eNOS may reflect beneficial effects of the tertiary structure of dyn-2 on binding kinetics which cannot be achieved by the PRD subdomain alone.

Upon determining the relevant domain of dyn-2 which is responsible for binding with eNOS, we next sought to determine the region of eNOS which is required for binding to dyn-2. For this purpose, we produced a series of in vitro $^{35}$S-labeled subdomain and deletion constructs of eNOS for use in the GST binding assay (Fig. 1A). In Fig. 2B, an autoradiograph is shown which depicts the radiolabeled eNOS full-length, reductase, and oxygenase domains, indicated by a single dominant protein band of the corresponding molecular size. GST, GST-dyn-2, or characterized GST dyn-2 subdomains, including GTPase, PHD, GED, and PRD, were incubated with $^{35}$S-eNOS full-length, reductase, or oxygenase domain proteins. Binding was assessed by SDS-PAGE and autoradiography. As seen in the representative autoradiograph in Fig. 2C, dyn-2 PRD binds the $^{35}$S-eNOS reductase domain, as well as $^{35}$S-eNOS full-length, but not the $^{35}$S-eNOS oxygenase domain. Note that binding of $^{35}$S-eNOS full-length or its subdomains is not detected with other GST-dyn-2 subdomains. To dissect further the relevant PRD binding region within the eNOS reductase domain, deletion constructs spanning the eNOS reductase domain were constructed for in vitro translation and then analyzed for their ability to bind dyn-2 PRD in the GST binding assay (Fig. 2A).

As seen in the autoradiograph in Fig. 2D, dyn-2 PRD binds eNOS 511–850 and eNOS 645–850 but not eNOS 511–645. These data suggest that dyn-2 PRD interacts within amino acids 645–850 within the eNOS reductase domain.

Because many dynamin-associated proteins bind by virtue of an SH3 domain-PRD interaction (24, 25, 28) and the eNOS sequence that binds dyn-2 PRD contains an SH3-like region at amino acids 767–823 (Fig. 2E), we next sought to determine whether dyn-2 PRD binding with eNOS could be competed by other SH3 domain-containing proteins. For this purpose, purified recombinant Fyn was added at increasing concentrations to the eNOS-GST-dyn-2 PRD binding assay, and bound proteins were assessed by Western blot analysis. Fig. 2F demonstrates that adding increasing amounts of Fyn protein competes off the binding of eNOS with dyn-2 PRD in a concentration-dependent manner (lanes 2–8), thereby inferring that the aforementioned SH3-like region within eNOS is likely responsible for dyn-2 PRD binding and also indicating that the binding of both Fyn and eNOS may reside on a similar polyproline sequence within the dyn-2 PRD region.

Next, to examine binding in the cellular context, we performed a mammalian two-hybrid screen using vectors encoding eNOS full-length, oxygenase, and reductase domains and reductase domain deletion fragments, fused with the GAL4 DNA binding domain, and vectors encoding dyn-2 or characterized dyn-2 subdomains, fused with an activation domain, derived from herpes simplex virus VP16 protein. Cells were transfected, and after 36 h lysates were prepared for CAT enzyme-linked immunosorbent assay and Renilla luciferase measurement. These analyses demonstrate a positive interaction between heterologously expressed eNOS full-length or eNOS reductase domain, and either dyn-2 or dyn-2 PRD, as assessed by a prominent increase in CAT expression (Table I). Furthermore, dyn-2 and dyn-2 PRD interact positively with eNOS full-length, eNOS 511–850, and eNOS 645–850, but not eNOS 511–645 (Table I). No substantive increase in CAT expression is detected upon expression of other dyn-2 subdomains with either full-length eNOS or the oxygenase domain of eNOS, providing specificity of effect. Additionally, transfection with empty pM and pVP16 vectors results in no significant CAT expression, whereas transfection with pM-53, which encodes mouse p53 protein, and pVP16-T, which encodes SV40 large T-antigen, a high affinity p53 binding partner, results in a level of CAT expression —2-fold that observed with the positive screens depicted on Table I (data not shown). Thus, these studies corroborate the results obtained in the GST binding assays and delineate the relevant subdomain interactions in the cellular context as well.

Dyn-2 PRD is sufficient for dyn-2-dependent potentiation of eNOS activity. We next explored the functional significance of the detected binding interaction between dyn-2 PRD and the eNOS reductase domain as it mechanistically relates to eNOS function. First we sought to determine whether dyn-2 PRD is responsible for dyn-2-dependent potentiation of eNOS activity. NOS activity assays were performed by assessing the ability of purified recombinant eNOS to convert L-arginine to L-citrulline and oxygenase domain function. eNOS is a bi-domain enzyme with the carboxyl-terminal reductase domain and interdomain electron transfer. As seen in Fig. 2C, dyn-2 PRD potentiates eNOS catalytic activity with a potency similar to dyn-2 full-length, whereas other dyn-2 subdomains do not influence NOS activity. Furthermore, as shown in Fig. 3B, dyn-2 PRD activation of eNOS occurs in a concentration-dependent manner and is dependent on an intact structure, as boiling of the GST fusion protein, prior to incubation with eNOS, renders dyn-2 PRD incapable of potentiating NOS activity. To gain further insight into the mechanism of dyn-2 PRD activation of eNOS, studies were performed with varying concentrations of calmodulin, a NOS-binding protein, which enhances eNOS reductase domain and interdomain electron transfer. As seen in Fig. 3C, dyn-2 PRD potentiates calmodulin-replete (100 nM) NOS activity, whereas dyn-2 PRD activation is abrogated in the presence of rate-limiting amounts of calmodulin (10 nM) and entirely abolished in the absence of exogenous calmodulin. These studies indicate that dyn-2 PRD is responsible for dyn-2-dependent potentiation of NOS activity and furthermore, that calmodulin is requisite for dyn-2 PRD potentiation of NOS activity.

Dyn-2 PRD selectively potentiates eNOS reductase domain function. eNOS is a bi-domain enzyme with the carboxyl-terminal reductase domain and amino-terminal oxygenase domain cooperating to allow electron transfer and ensuing NO generation (1). Reductase domain function is comprised in part by a series of electron transfer steps, including electron transfer from NADPH into the flavin groups via FAD, with subsequent electron transfer to the heme group within the oxygenase domain (1). The activity of the eNOS oxygenase domain can also be assessed independently of reductase domain function by measuring the ability of recombinant eNOS to generate nitrite from the reaction intermediate, NOHA, NOHA (14, 18, 19). To determine how dyn-2 potentiates eNOS activity, we examined the effects of GST-
Dyn-2 PRD on some of these individual NOS reductase and oxygenase subdomain functions. We first examined the influence of dyn-2 full-length and dyn-2 PRD on eNOS oxygenase domain function. In these experiments, increasing amounts of recombinant Fyn were also added to the reaction mix. Binding was assessed by SDS-PAGE and autoradiography of dried gels, Western blot analysis, and Coomassie staining of gels, as indicated. A, physical maps of the eNOS constructs utilized for mapping of the domain contributing to the interaction between eNOS and dyn-2. The numbers shown on the top of each box correspond to amino acid residues. B, autoradiograph of SDS-PAGE demonstrates the purity and specificity of $^{35}$S-eNOS full-length, reductase, and oxygenase subdomains as assessed by a single dominant band of the correct molecular size for each protein. C, a representative SDS-PAGE autoradiograph shows that GST-dyn-2 PRD binds the $^{35}$S-eNOS full-length (top panel) and $^{35}$S-eNOS reductase domain (middle panel) but not the $^{35}$S-eNOS oxygenase domain (bottom panel). Note that other dyn-2 subdomains do not bind $^{35}$S-eNOS full-length or eNOS subdomains. The radiograph is representative of three independent experiments that yielded comparable results. D, a representative SDS-PAGE autoradiograph shows that GST-dyn-2 PRD binds $^{35}$S-eNOS 511–850 (lane 2) and $^{35}$S-eNOS 645–850 (lane 4) but not eNOS 511–645 (lane 6). Lanes 1, 3, and 5 depict the input of the respective $^{35}$S-eNOS fragments in the binding assay. E, the sequence of the putative SH3 domain of eNOS contained within the region that binds with dyn-2 PRD is shown in comparison with SH3 domains from phospholipase Cγ, Nck, and Fyn (white boxes denote identical amino acids residues between eNOS and the SH3 domains of phospholipase Cγ, Nck, and Fyn). Comparison was made using SeqWeb Version 1.2. F, a representative Western blot using eNOS monoclonal antibody and Fyn monoclonal antibody is shown and demonstrates that when added at increasing concentrations, recombinant Fyn competes with purified recombinant eNOS protein for binding with GST-dyn-2 PRD (lanes 2–8). Lane 8 indicates the maximum Fyn input in the binding reactions. The bottom panel is a Coomassie-stained gel demonstrating similar levels of GST-dyn-2 PRD in each binding reaction. The experiment was repeated twice and yielded similar results.
eNOS reductase domain activity is potentiated by dyn-2 full-length as well as dyn-2 PRD but not significantly influenced by other dyn-2 subdomains (Fig. 4B). Furthermore, the effect of dyn-2 PRD on eNOS-mediated cytochrome c reduction occurs in a concentration-dependent manner as assessed by varying the concentration of dyn-2 PRD added to the assay (Fig. 4C). Next, to examine the influence of dyn-2 PRD on the more proximal transfer of electrons from NAPDH to FA, the influence of dyn-2 PRD was examined on electron transfer to the artificial electron acceptor, FeCN. In these experiments, in contradistinction to its stimulatory effect on cytochrome c reduction, dyn-2 PRD, at varying molar concentrations does not influence FeCN reduction in a significant manner (Fig. 4D). These studies indicate that dyn-2 potentiates eNOS function, at least in part, through selective effects on the reductase domain of eNOS, with most prominent influence on intracellular electron transfer.

**DISCUSSION**

Post-translational mechanisms of eNOS activation are an area of active investigation. In this regard, eNOS catalytic function is influenced by specific events, including phosphorylation, acylation, and protein interactions (2, 4–6, 37–39). In support of the latter concept, specific proteins have been identified which bind eNOS and regulate enzyme function (9–11, 40, 41). The current study delineates the eNOS binding and activation mechanisms of one of these proteins, dyn-2, using purified proteins (12). We have utilized complementary approaches to establish that dyn-2 PRD and eNOS reductase domain contain the cognate binding sequences that are responsible for mediating the binding interaction between dyn-2 and eNOS. These include in vitro binding assays, utilizing appropriate GST-fused dyn-2 subdomains incubated with both recombinant eNOS protein derived from *E. coli* and radiolabeled in vitro transcribed subdomain peptides, as well as competition studies using the SH3-containing protein, Fyn. Corroborative evidence for interaction between these specific subdomains is demonstrated by a mammalian two-hybrid screen, thereby providing assurance that these subdomains can interact in cells as well. Further correlative evidence is provided by the binding affinity detected between eNOS and dyn-2 PRD, which is relatively similar to that observed between the eNOS and the dyn-2 full-length protein. Detection of PRD as the eNOS binding domain is not entirely unexpected because dyn-2 PRD is responsible for many of the protein interactions with which dynamins are associated, including AP-2, Grb2, phospholipase Cγ, and phosphatidylinositol 3-kinase (24, 25, 28). Proline-rich sequences are a common ligand preference for a variety of protein interaction domains (42), and many dynamin associ-

| TABLE I | eNOS reductase domain interacts with dyn-2 PRD in mammalian two-hybrid screen |
|---------|--------------------------------------------------|
| eNOS-pM-GAL4 fusion | Dyn-2 full length | GTPase | GED | PHD | PRD |
| eNOS full-length | 57.7 ± 5.4* | 2.7 ± 0.4 | 3.1 ± 0.2 | 3.1 ± 0.2 | 40.9 ± 1.2* |
| eNOS oxygenase domain (1–511) | 1.4 ± 0.1 | 3.2 ± 0.2 | 0.0 ± 0.0 | 0.0 ± 0.0 | 2.3 ± 0.2 |
| eNOS reductase domain (511–204) | 56.5 ± 1.3* | 4.5 ± 0.3 | 0.22 ± 0.02 | 0.0 ± 0.0 | 49.8 ± 7.4* |
| eNOS reductase domain (511–645) | 55.4 ± 2.4* | 1.7 ± 0.1 | 0.1 ± 0.1 | 2.8 ± 0.4 | 39.6 ± 1.1* |
| eNOS reductase domain (645–850) | 7.6 ± 0.5 | 5.8 ± 0.4 | 0.2 ± 0.0 | 6.7 ± 0.5 | 8.8 ± 1.0 |
| eNOS reductase domain (645–850) | 37.9 ± 2.2* | 9.7 ± 0.7 | 2.6 ± 0.5 | 2.9 ± 0.4 | 30.9 ± 2.8* |

**Fig. 3.** Dyn-2 PRD is the requisite subdomain for dyn-2-dependent potentiation of eNOS activity. The activity of *E. coli*-derived purified recombinant eNOS was examined after incubating recombinant eNOS protein with GST-dyn-2 full-length or alternatively, dyn-2 subdomains (GTPase, PHD, GED, and PRD). NOS activity was assessed by measuring the L-NAME-inhibited conversion of L-arginine to L-citrulline. A, dyn-2 PRD potentiates eNOS catalytic activity in a magnitude similar to dyn-2 full-length, whereas other dyn-2 subdomains do not influence NOS activity (*, p < 0.05 compared with 0.05 compared with other groups; n = 3 separate experiments, each performed in duplicate).
Dyn-2 PRD and eNOS

Fig. 4. Dyn-2 PRD potentiates eNOS reductase domain function in a selective manner. The influence of GST dyn-2 full-length and characterized dyn-2 subdomains on eNOS subdomain function was examined using complementary spectrophotometric assays. A, the influence of GST-dyn-2, GST-dyn-2 PRD, or GST alone on eNOS oxygenase domain was examined by measuring the ability of recombinant eNOS protein to convert DOHA to nitrite. Nitrite was measured using the Griess reaction. Neither dyn-2 full-length (FL) nor dyn-2 PRD influences the oxygenase domain function of eNOS (n = 3 separate experiments, each performed in triplicate). B, the cytochrome c reducing capacity of recombinant eNOS protein was examined after incubation of eNOS with GST-dyn-2 subdomains or GST alone. Cytochrome c reduction by eNOS was examined after incubation of eNOS performed in duplicate. C, the influence of GST-dyn-2, GST-dyn-2 PRD, or GST alone on eNOS reductase domain function was examined using complementary spectrophotometric assays. D, the influence of GST-dyn-2, GST-dyn-2 PRD, or GST alone on eNOS reductase domain function was examined using complementary spectrophotometric assays.

In summary, these studies indicate that dyn-2 PRD and the eNOS reductase domain contain the proline binding sequence responsible for the interaction between eNOS and dyn-2. Our deletion analysis indicates that, of these, the SH3-like region at amino acids 767–823 is likely responsible for dyn-2 PRD binding because this region lies within the reductase domain fragment that binds dyn-2 PRD by GST binding assay and mammalian two-hybrid screen. Further evidence for this inference is provided by our data demonstrating the ability of the SH3 domain-containing protein, Fyn, to compete quantitatively with eNOS for binding with dyn-2 PRD. These competitive binding data also suggest that Fyn and eNOS may bind a common polyproline sequence within the dyn-2 PRD region.

How does dyn-2 PRD influence eNOS function? First, influences on the eNOS reductase domain are likely to confer effects on overall NOS enzyme activity as the rate of electron transfer across the reductase domain has been postulated to be the rate-limiting step in NO formation from eNOS (13, 44, 45). Consistent with this concept, we find a direct correspondence among the site of dyn-2 binding on eNOS within the reductase domain, its ability to potentiate reductase domain function, and ensuing augmentation of NOS activity. Our studies also indicate that dyn-2 PRD potentiates calmodulin-replete NOS activity only. Thus, dyn-2 does not influence the integral calmodulin requirements in the eNOS pathway. This is evidenced by abolition of the NOS stimulatory effects of dyn-2 PRD upon depletion of calmodulin. These data also suggest that calmodulin and dyn-2 are unlikely to compete for a similar binding site on eNOS. The reductase domain of eNOS catalyzes several distinct electron transfer steps between NADPH, FAD, FMN, and ultimately to the heme group. The selective influence of dyn-2 PRD on cytochrome c reduction in the absence of a prominent effect on FeCN reduction suggests that dyn-2 PRD likely influences intraflavin electron transfer rather than electron transfer into the flavins from NADPH. This is because electron transfer to FeCN proceeds from FAD, whereas electron transfer to cytochrome c is preferential from FMN (15, 17). These functional observations are consistent with the mapping of the dyn-2 PRD binding sequence to a region overlapping with the eNOS FAD binding domain.

In the present studies, dyn-2 PRD is sufficient for dyn-2-dependent NOS function. These studies add to a series of recent observations, which strongly implicate dyn-2, and in some instances, the dyn-2 PRD domain, as a mediator of cell signaling events (46). Indeed, dyn-2 signaling has been implicated in the regulation of diverse cell signaling pathways including mitogen-activated protein kinase and more recently in the transcriptional regulation of p53 and downstream apoptosis (27, 46). Furthermore, dyn-2 PRD-protein interactions are also important in serving the more established function of dyn-2 as it relates to vesicle scission events, as evidenced by the demonstration of dyn-2 PRD interaction with the actin-binding protein cortactin (32). Thus dyn-2 PRD appears responsible for mediating a variety of dynamin-dependent functions by virtue of direct protein binding events.
reductase domain of calmodulin-replete eNOS at least in part by influencing electron transfer between the flavins. Together these studies expand our current understanding of the molecular mechanisms underlying the regulation of eNOS.

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