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Targeting of Endothelial Nitric-oxide Synthase to the Cytoplasmic Face of the Golgi Complex or Plasma Membrane Regulates Akt- *Versus* Calcium-dependent Mechanisms for Nitric Oxide Release*

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The heterogeneous localization of endothelial nitric-oxide synthase (eNOS) on the Golgi complex *versus* the plasma membrane has made it difficult to dissect the regulation of each pool of enzyme. Here, we generated fusion proteins that specifically target the plasma membrane or cytoplasmic aspects of the Golgi complex and have assessed eNOS activation. Plasma membrane-targeted eNOS constructs were constitutively active, phosphorylated, and responsive to transmembrane calcium fluxes, yet were insensitive to further activation by Akt-mediated phosphorylation. In contrast, *cis*-Golgi complex-targeted eNOS behaved similarly to wild-type eNOS and was less sensitive to calcium-dependent activation and highly responsive to Akt-dependent phosphorylation compared with plasma membrane versions. In plasma membrane- and Golgi complex-targeted constructs, Ser^{1179} is critical for NO production. This study provides clear evidence for functional roles of plasma membrane- and Golgi complex-localized eNOS and supports the concept that proteins thought to be regulated and to function exclusively in the plasma membrane of cells can indeed signal and be regulated in internal Golgi membranes.

Within the vascular endothelium, nitric oxide (NO) is generated via the enzyme endothelial nitric-oxide synthase (eNOS).[1] This highly labile and reactive gas plays a key role in regulating cardiovascular homeostasis, influencing systemic and pulmonary blood pressure, vascular remodeling, and angiogenesis. Given the important and pervasive action of endothelium-derived NO in the cardiovascular system, it is not surprising that the activity of eNOS is highly regulated in a temporal and spatial manner (1). eNOS was originally characterized as a calcium/calmodulin-dependent enzyme (2–4), and although this is the primary means by which eNOS activity is controlled, discrepancies between the levels of eNOS protein, the amount of NO released, calcium regulation of the enzyme, and the impaired function of mislocalized eNOS have led to the discovery of additional post-translational control mechanisms regulating eNOS activity. It is now generally accepted that eNOS activity is also influenced directly by regulated protein-protein interactions and phosphorylation and indirectly by fatty acylation, which targets the enzyme to specific intracellular domains (1, 5, 6). Activation of endothelial cells in response to vascular endothelial growth factor (7, 8), sphingosine-1-phosphate (9, 10), estrogen (11, 12), insulin (13), and shear stress (14) results in the Akt-dependent phosphorylation of Ser^{1179} on eNOS. Although there is increasing evidence that other phosphorylation sites (Ser^{116}, Thr^{497}, Ser^{617}, and Ser^{635}) (15–18) contribute to temporal regulation of eNOS function, mutation of Ser^{1179} prevents both Akt-mediated phosphorylation and NO release, and dominant-negative Akt reduces endothelium-dependent vascular responses *in vitro* and *in vivo* (7, 14, 19), thus highlighting the importance of this regulatory site. The ability of both Akt-phosphorylated and S1179D eNOS (a constitutively active allele in which Ser^{1179} is mutated to an aspartate reside) to release more NO in the absence of a stimulus has led to the proposal that phosphorylation of Ser^{1179} mediates the calcium-independent activation of eNOS (7, 14, 20).

eNOS is modified by post-translational myristoylation of Gly^{2} and palmitoylation of Cys^{15} and Cys^{26} (21–23). These N-terminal fatty acid modifications target eNOS to the Golgi complex and to cholesterol-enriched domains of the plasma membrane, including caveolae and "lipid rafts" (24–26). Whether *in situ* within blood vessels or in cultured endothelial cells, the relative proportion of eNOS in the Golgi complex *versus* the plasma membrane is variable and can depend on the vascular bed, species, and state of confluency (27–29). The *N*-myristoylation site mutant of eNOS is catalytically competent, remains cytosolic, and displays reduced NO release. Biochemically, this is due to a reduction in Ser^{1179} phosphorylation (30–32). Phosphorylation of eNOS at Ser^{1179} has been observed in the Golgi complex and in the plasma membrane of endothelial cells; however, due to the presence of eNOS in both locations, the individual functions of Golgi *versus* plasmalemmal eNOS, if any, are not yet known and are matter of mere speculation (30). Therefore, the goal of this study was to determine the individual function of eNOS in the cytoplasmic aspect of the Golgi complex and plasma membrane using molecular targeting with organelle-restricted "zip codes" to deliver functional eNOS to specific intracellular sites and to examine whether eNOS targeted to endomembranes is functionally responsive to extracellular stimuli.

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[1] The abbreviations used are: eNOS, endothelial nitric-oxide synthase; WT, wild-type.

MATERIALS AND METHODS

Generation of eNOS Targeting Proteins

The cytosolic myristoylation site mutant (G2A eNOS) is catalytically identical to the wild-type (WT) enzyme (33) and was used as an enzymatic scaffold for attaching the different targeting sequences as outlined below.

Golgi Complex-targeted Constructs

GRIP-eNOS—The GRIP domain (~42 amino acids) targets cargo to a distinct population of *trans*-Golgi tubulovesicular membranes. This domain is shared by a family of coiled-coil peripheral membrane proteins, including p230, golgin-97, and golgin-245 (34). A region of golgin-97 containing the GRIP domain (87 amino acids) was isolated from human umbilical vein endothelial cell mRNA; fused in-frame to G2A eNOS using PCR overlap extension with primers 5′-TGT GCC TCG AGC GGG GCC ACA TGT T-3′ (sense), 5′-CCA GAC ACC CCC GGC CCC GTC ACG AAT AAC ACT GAC-3′ (middle sense), 5′-GTC AGT GTT ATT CGT GAC GGG GCC GGG GGT GTC TGG-3′ (middle sense), and 5′-ACT AGT TGA GTC TAG ACT AGG ACC ATG GTA TCC GAG GG TT-3′ (antisense); and encodes the GRIP domain (VTNNTDLTDAREINFEYLKHVVLKFMSCRESEAFHLIKAVSVLLNFSQEEENMLKETLEYKMSWFGSKPAPKGSIRPSISNPRIPWS).

Syn17-eNOS—The length of the transmembrane domain of syntaxin-3 determines membrane association and subcellular targeting (35). The truncated transmembrane domain of syntaxin-3 (referred to as Syn17; 18 amino acids, KLIIIIVLVVVLLGILAL) that targets the *cis*/medial-Golgi complex was fused in-frame to the C terminus of G2A eNOS using primers 5′-TGT GCC TCG AGC GGG GCC ACA TG TT-3′ (sense) and 5′-AAA TCT AGA TCT AGA TCA AAT CAA TGC TAA AAT GCC CAG CAA CAC AAC TAC TAG CAC AAT GAT AAT TAT CAA TTT GGG GCC GGG GGT GTC TGG GC-3′ (antisense).

Plasma Membrane-targeted Constructs

CAAX-eNOS—The K-Ras polybasic (polylysine) domain containing the C*AAX* (where *A* is aliphatic amino acid) motif, which undergoes farnesylation, proteolysis of *AAX*, and methyl esterification (GKKKKKKSKTKCVIM), was fused in-frame to the C terminus of G2A eNOS using primers 5′-TGT GCC TCG AGC GGG GCC ACA TGT T-3′ (sense) and 5′-TAT CTA GAT CTA GAT TAC ATA ATT ACA CAC TTT GTC TTT GAC TTC TTT TTC TTC TTT TTA CCG GGG CCG GGG GTG TCT GGG CCG GG-3′ (antisense) (36).

Syn25-eNOS—A region of syntaxin-3 containing the full-length plasma membrane-targeting domain (27 amino acids, KLIIIIVLVVVLLGILALIIGLSVGLN) was fused in-frame to the C terminus of G2A eNOS using primer 5′-ATC TAG ATC TAG ATC AAT TCA GCC CAA CGG AAA GTC CAA TAA TCA ATG CTA AAA TGC CCA GCA ACA CAA CTA CTA GCA CAA TGA TAA TTA TCA ATT TGG GGC CGG GGG TGT CTG GGC-3′ (antisense).

Cell Culture Conditions and Transfection

COS-7 cells were grown in Dulbecco's modified Eagle's medium containing 100 units/ml penicillin, 100 mg/ml streptomycin, and 10% (v/v) fetal calf serum. For transfection, COS-7 cells were seeded at a density of 1.3×10^6 cells/100-mm dish and transfected the next day with the cDNAs for WT eNOS, G2A eNOS, GRIP-eNOS, C*AAX*-eNOS, Syn17-eNOS, Syn25-eNOS, Flk, and Akt using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions.

Western Blotting

Cells were washed twice with phosphate-buffered saline and lysed on ice in 50 mM Tris-HCl (pH 7.5), 1% (v/v) Nonidet P-40, 10 mM NaF, 1 mM vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, and 10 mg/ml leupeptin, and lysates were transferred to an Eppendorf tube and rotated for 45 min at 4 °C. Lysates were homogenized in a Dounce homogenizer (50 strokes), and insoluble material was removed by centrifugation at $12{,}000 \times g$ for 10 min at 4 °C, size-fractionated by SDS-PAGE, and Western-blotted as described previously (7).

Immunofluorescence

COS-7 cells were transfected as described above and plated onto sterile coverslips. The cells were then fixed in 1:1 acetone/methanol for 3 min at −20 °C and rinsed twice with phosphate-buffered saline plus 0.1% bovine serum albumin for 5 min at room temperature. The cells were incubated with 5% goat serum in phosphate-buffered saline plus 0.1% bovine serum albumin for 30 min at room temperature, followed by incubation for 2 h with primary antibody (polyclonal or monoclonal) at room temperature. Texas Red-labeled anti-rabbit (diluted 1:100) or fluorescein isothiocyanate-labeled anti-mouse (diluted 1:100) secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was incubated for 1 h at room temperature. Slides were mounted with Slowfade (Molecular Probes, Inc., Eugene, OR), and cells were observed with an inverted Zeiss microscope fitted with a Bio-Rad MRC 600 confocal imaging system. Antibodies for eNOS were obtained from BD Biosciences (anti-eNOS monoclonal antibody), and those for GM130 were from G. Warren (Yale University).

NO Release

Medium (100 µl) containing NO (primarily NO_2^-) was ethanol-precipitated to remove proteins and refluxed in sodium iodide/glacial acetic acid to convert NO_2^- to NO. NO was quantitated via specific chemiluminescence following reaction with ozone (Sievers). Net NO_2^- was calculated after subtracting unstimulated basal release (7).

NOS Activity Measurements

The activity of WT and targeted eNOS was determined in detergent-solubilized lysates of transfected COS-7 cells by measuring the conversion of [^{14}C]arginine to [^{14}C]citrulline as described previously (7).

Statistical Analysis

NO release data are expressed as means ± S.E. Comparisons were made using two-tailed Student's *t* test or analysis of variance with a post-hoc test where appropriate. Differences were considered as significant at $p < 0.05$.

RESULTS

Generation and Characterization of eNOS Targeting Constructs—A cytosolic mutant of eNOS (G2A) that cannot be *N*-myristoylated was used to generate eNOS fusion proteins that specifically target the Golgi complex or plasma membrane (Fig. 1). Previous work has shown that G2A eNOS retains full catalytic ability *in vitro*; however, the stimulated release of NO from intact cells expressing this mutant is impaired because the enzyme fails to target the Golgi complex and plasmalemmal rafts/caveolae and is not efficiently phosphorylated by Akt at Ser^{1179} (30, 33, 37). By exploiting these properties of G2A eNOS, we generated Golgi complex- and plasma membrane-targeted eNOS to assess the functional importance of subcellular targeting (Fig. 1).

To determine whether the eNOS fusion proteins target their intended destinations, indirect immunofluorescence for eNOS and GM130, a resident peripheral Golgi protein, was performed in transfected COS cells. As shown in Fig. 2, WT eNOS was present in both the plasma membrane (*WT-eNOS*, *left panel*, *arrows*) and the Golgi complex (*arrowhead*), co-localizing with the Golgi marker GM130 (*right panel*, *arrowhead*), whereas G2A eNOS was diffusely distributed throughout the cell (*G2A-eNOS*, *left panel*) and did not specifically co-localize with GM130 (*right panel*). Targeting of eNOS to the Golgi complex with the GRIP domain was confirmed by the presence of eNOS concentrated in a perinuclear pattern (*GRIP-eNOS*, *left panel*), but it was also found in a pattern reminiscent of post-Golgi vesicles with the absence of plasma membrane staining. GRIP-eNOS partially co-localized with the Golgi marker GM130 (*right panel*). Similarly, fusion of eNOS with the syntaxin-3 *cis*/medial-Golgi complex-targeting motif (Syn17-eNOS) demonstrated Golgi targeting (*S17-eNOS*, *left panel*) and partial co-localization with GM130 and no plasma membrane staining (*right panel*). Fusion of eNOS to the full-length transmembrane domain of syntaxin-3 (Syn25-eNOS) resulted in dramatic plasma membrane staining, with minor amounts present in the Golgi complex (*S25-eNOS*, *left panel*). Membrane targeting of eNOS with the C*AAX* motif derived from K-Ras (C*AAX*-eNOS) also showed the majority of eNOS in the plasma membrane (*CAAX-eNOS*, *left panel*). Thus, using these targeting motifs, eNOS was targeted to the peripheral aspect of the Golgi network (GRIP-eNOS and Syn17-eNOS) or the plasma membrane (Syn25-eNOS and C*AAX*-eNOS).

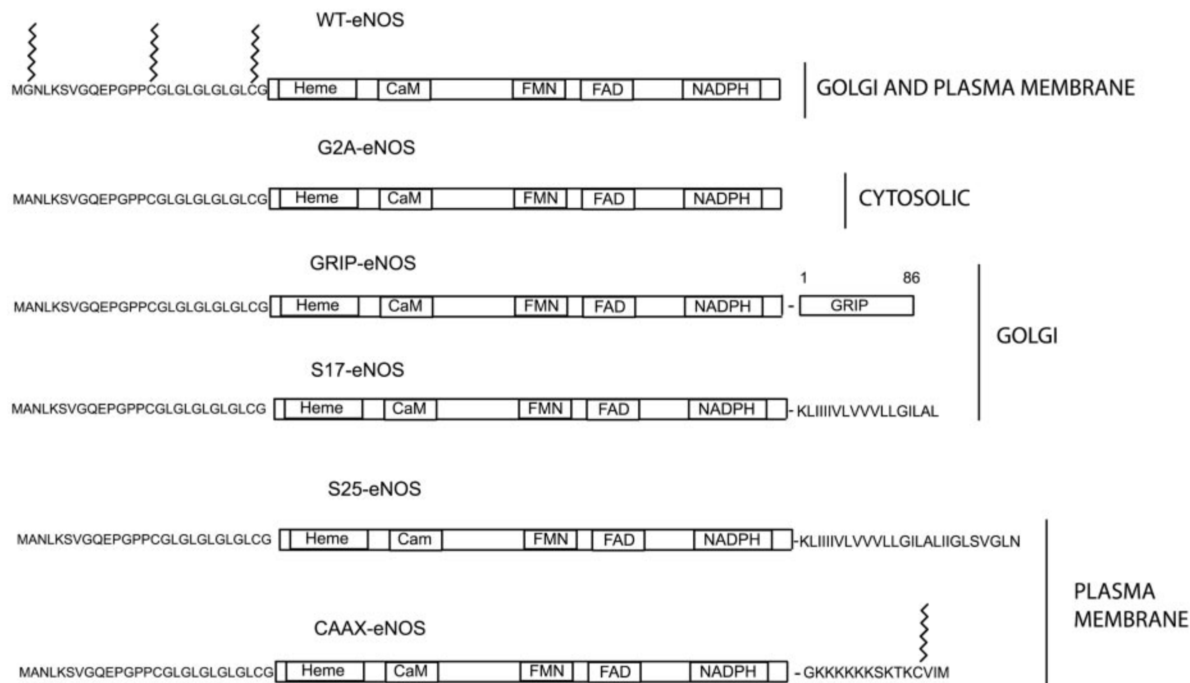

FIG. 1. **Generation of subcellular eNOS targeting fusion proteins.** WT eNOS is myristoylated at Gly[2] and palmitoylated at Cys[15] and Cys[26], post-translational modifications that target the enzyme to both the Golgi complex and plasma membrane. G2A eNOS lacks the fatty acid acylation required for membrane association and is dispersed throughout the cytosol. GRIP-eNOS, Syn17-eNOS, Syn25-eNOS, and C*AAX*-eNOS are C-terminal fusion proteins generated from mistargeted, catalytically competent G2A eNOS. *CaM*, calmodulin.

Previous studies have shown that WT and G2A eNOS have similar catalytic abilities and cofactor requirements (38, 39) despite the inability of mislocalized G2A eNOS to produce NO after stimulation of cells with calcium-mobilizing agonists (33, 37). Therefore, we measured the catalytic competency of the newly generated eNOS fusion proteins *in vitro* using the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline as an index of NOS activity in detergent-solubilized COS cell extracts. COS cells do not contain any endogenous NOS isoform, and all activity measurements were eliminated by the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester hydrochloride. The activity of GRIP-eNOS was slightly greater than that of WT eNOS (89.7 ± 2.7 and 72.8 ± 5.5 pmol of L-citrulline/mg/min, respectively; $n = 3$). In a separate series of transfectants, Syn17-eNOS, Syn25-eNOS, and C*AAX*-eNOS exhibited slightly lower catalytic activities compared with WT eNOS (25.4 ± 0.6, 20.8 ± 1.5, 14.3 ± 0.5, and 32.9 ± 1 pmol of L-citrulline/mg/min, respectively; $n = 3$). These differences in activity were not due to expression differences as assessed by semiquantitative Western blotting of the same extracts.

Plasma Membrane Targeting of eNOS Results in Marked NO Release due to Constitutive Phosphorylation of Ser[1179]—To determine the relevance of intracellular location to eNOS function in intact cells, COS cells were transfected with the various eNOS constructs, and basal NO release was determined by NO-specific chemiluminescence. Cells were subsequently lysed and immunoblotted for total eNOS or phosphorylated eNOS species using phosphorylation state-specific antibodies (Ser(P)[1179], Thr(P)[497], Ser(P)[617], Ser(P)[635]). As shown in Fig. 3, non-acylated cytosolic G2A eNOS displayed both significantly reduced NO release (*A*, *graph*) and hypophosphorylation of Ser[1179] (*A*) Thr[497] (*B*), and Ser[617] and Ser[635] (*C*) compared with WT eNOS (*upper gels*). On the other hand, the plasma membrane-targeted forms of eNOS fusion proteins (Syn25-eNOS and C*AAX*-eNOS) produced substantially more basal NO than did WT eNOS, with equivalent levels of eNOS protein expression. As shown in Fig. 3*A* (*upper* and *lower gels*), the level of Ser[1179] phosphorylation was also much greater in the plasma membrane-targeted forms of eNOS relative to the WT enzyme. Localization of eNOS to the peripheral aspects of the Golgi network produced divergent results. Restricted localization of eNOS to the *trans*- and post-Golgi vesicles via the GRIP targeting motif (GRIP-eNOS) resulted in reduced basal NO release compared with the WT enzyme. In contrast, fusion of eNOS to the syntaxin-3 *cis*-Golgi complex-targeting motif (Syn17-eNOS) produced equivalent amounts of basal NO compared with the WT enzyme. These changes in NO release did not strictly correlate with basal phosphorylation of Ser[1179] since GRIP-eNOS and Syn17-eNOS phosphorylation was comparable with WT eNOS phosphorylation, but was again greater than G2A eNOS phosphorylation.

We next investigated the relative degree of eNOS phosphorylation of the other regulatory phosphorylation sites. Thr[497] phosphorylation is important for the fidelity of eNOS to produce NO *versus* superoxide since phosphorylation promotes NO release, whereas dephosphorylation promotes superoxide anion production (18). Phosphorylation of Ser[617] and Ser[635] regulates temporal aspects of calcium/calmodulin regulation of the enzyme (17, 40). As shown in Fig. 3*B*, WT eNOS and plasma membrane-targeted eNOS (Syn25-eNOS and C*AAX*-eNOS) exhibited enhanced Thr[497] phosphorylation relative to G2A eNOS and the Golgi complex-targeted eNOS constructs. The phosphorylation of Ser[617] was similarly distributed, with the greatest degree of phosphorylation observed with the WT and plasma membrane-targeted eNOS constructs. Low levels of Ser[617] phosphorylation were observed with cytosolic G2A eNOS and eNOS fusion proteins that target the Golgi network (GRIP-eNOS and Syn17-eNOS). GRIP-eNOS displayed the highest level of Ser[635] phosphorylation, followed by WT eNOS and plasma membrane-targeted eNOS (C*AAX*-eNOS and Syn25-eNOS). Syn17-eNOS (*cis*-Golgi complex-targeted) was found to have significantly lower phosphorylation of Thr[497], Ser[635], and Ser[617] (Fig. 3, *B* and *C*).

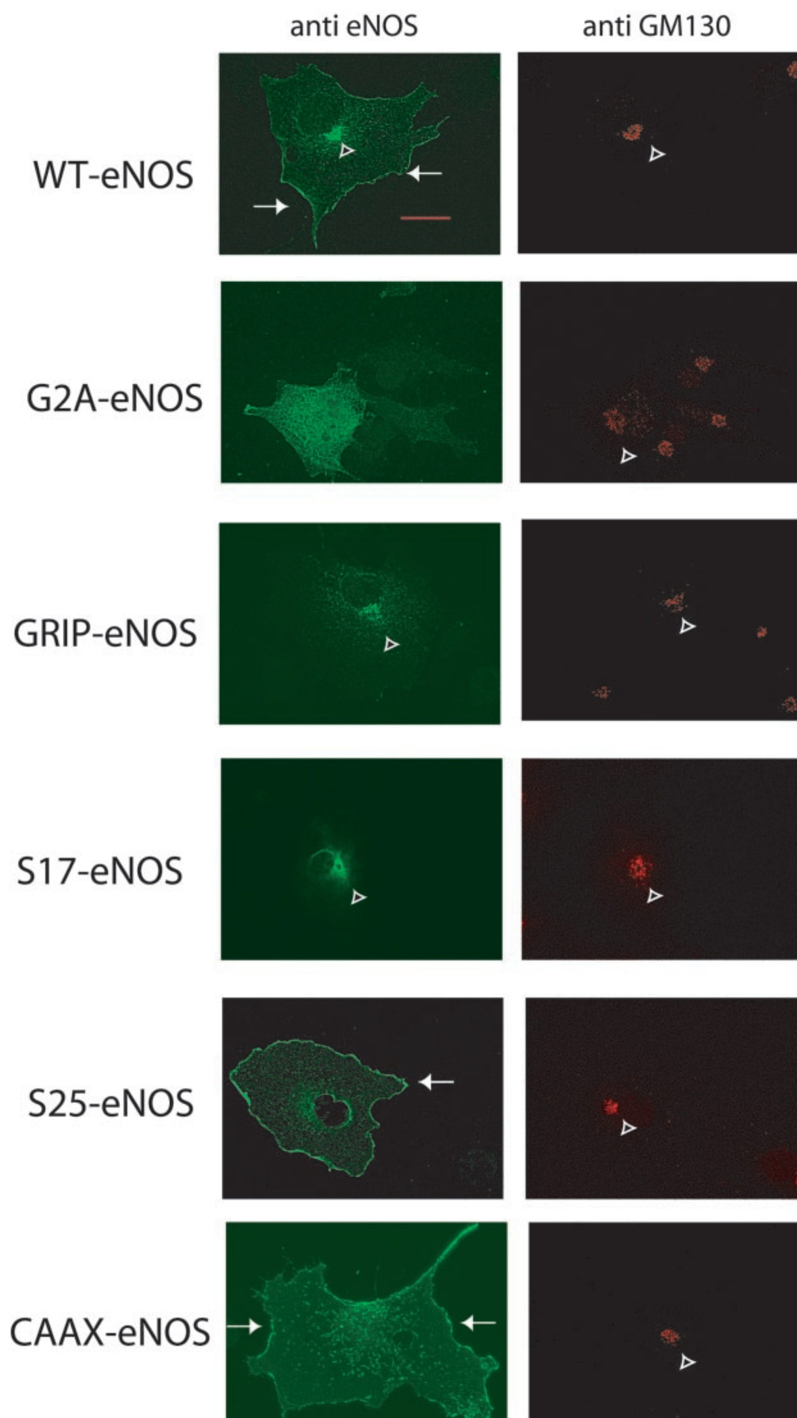

FIG. 2. **Localization of WT eNOS, G2A eNOS, and eNOS fusion proteins.** COS cells were transfected with cDNAs encoding the various eNOS constructs, and cells were immunolabeled with antibodies for total eNOS (*left panels*; *green*) and the resident Golgi protein GM130 (*right panels*; *red*). *WT-eNOS*, WT eNOS was found in both the perinuclear Golgi complex (*arrowheads*) and the plasma membrane (*arrows*) (*left panel*). Perinuclear staining of WT eNOS co-localized with the Golgi marker GM130 (*right panel*; *red*). *G2A-eNOS*, G2A eNOS was distributed throughout the cytoplasm. *GRIP-eNOS* and *S17-eNOS*, shown is the Golgi complex-restricted expression of the Golgi complex-targeted eNOS fusion proteins (GRIP-eNOS and Syn17-eNOS, respectively) and co-localization with GM130. *S25-eNOS* and *CAAX-eNOS*, the contrasting expression of plasma membrane-targeted eNOS (Syn25-eNOS and C*AAX*-eNOS, respectively) is highly evident in the plasma membrane, with limited endomembrane staining.

Plasma Membrane-targeted eNOS Is Highly Sensitive to Transmembrane Calcium Fluxes—Thapsigargin, an inhibitor of SERCA, elevates intracellular calcium through store depletion, which in turn triggers calcium influx via capacitive calcium entry (41). The elevation of intracellular calcium is a key determinant of eNOS activation via the increased association of calcium-activated calmodulin. As shown in Fig. 4*A*, thapsigargin evoked release of substantially more NO from COS-7 cells transfected with plasma membrane-targeted forms of eNOS (Syn25-eNOS and C*AAX*-eNOS) compared with cells expressing WT eNOS or Golgi complex-restricted eNOS (GRIP-eNOS and Syn17-eNOS). Compared with the WT enzyme, calcium-dependent activation of cytosolic G2A eNOS resulted in the production of significantly less NO, whereas both *trans*-Golgi-localized GRIP-eNOS and *cis*/medial-Golgi targeting with Syn17-eNOS were not significantly different from that with WT eNOS.

Golgi Targeting with Syn17-eNOS Is Required for Akt-dependent Activation of eNOS—As stated previously, eNOS can be activated via Akt-dependent phosphorylation of Ser^{1179} (7, 14). As shown in Fig. 4*B*, cotransfection of WT eNOS with cDNA for Akt-1 resulted in the increased phosphorylation of Ser^{1179} (*upper gels*) and enhanced production of NO (*graph*). Previous work has shown that this effect is abolished if Ser^{1179} is mutated to alanine (7, 14). Fatty acylation and subcellular targeting are required for full activation, as Akt stimulated significantly less NO production (*graph*) and Ser^{1179} phosphorylation (*upper gels*) with cytosolic G2A eNOS *versus* the WT enzyme. Although a significant increase in NO release was observed in the presence of Akt, this was not greater than the

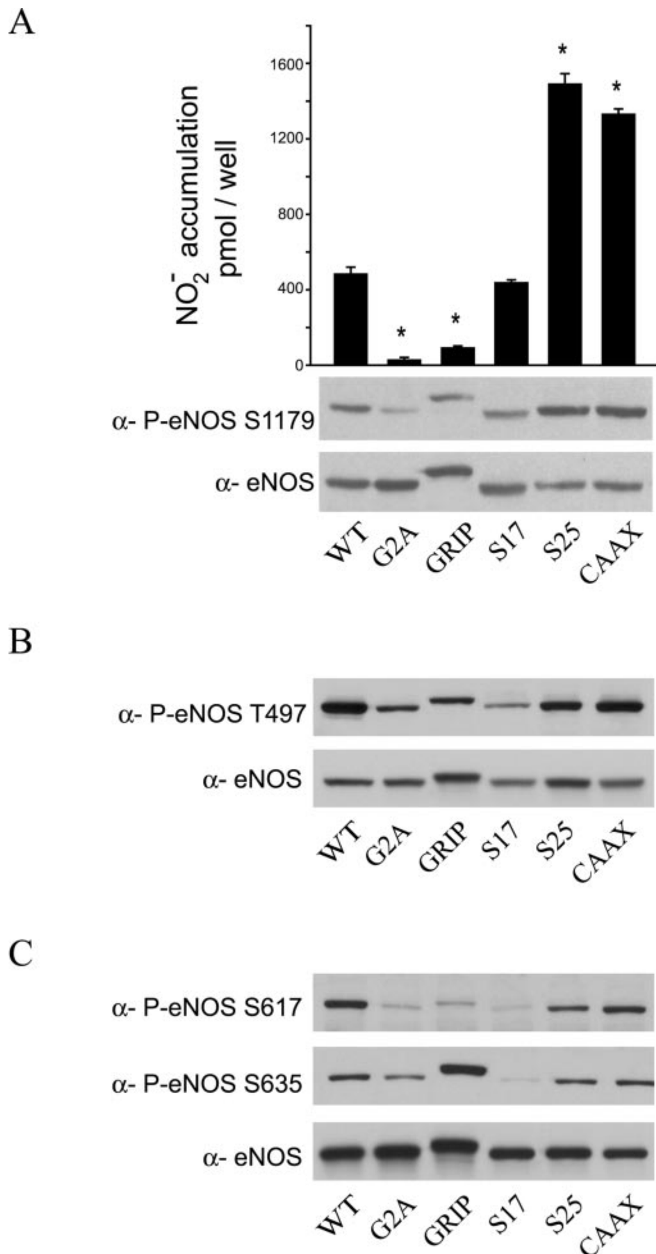

FIG. 3. **The basal NO release and phosphorylation state of plasma membrane-targeted eNOS fusion proteins are greater compared with those of Golgi complex-targeted forms.** WT eNOS, G2A eNOS, and Golgi complex- and plasma membrane-targeted eNOS fusion proteins were transfected into COS cells, and basal NO release was determined via NO-specific chemiluminescence over 24 h (*graph*). There was no significant difference between the various eNOS constructs at the level of total protein/well. Cell lysates were immunoblotted for changes in the phosphorylation state of Ser^{1179} (*A*), Thr^{497} (*B*), and Ser^{617} and Ser^{635} (*C*) (*upper gels*) relative to the level of total eNOS (*lower gels*). The data are presented as means ± S.E. ($n = 5$). *, $p < 0.05$ *versus* the WT enzyme. *S17*, Syn17-eNOS; *S25*, Syn25-eNOS.

unstimulated levels for the WT enzyme (Fig. 4*B*, *graph*). Transfection with Akt did not stimulate statistically significant NO production from GRIP-eNOS; however, restricted *cis*/medial-Golgi localization with Syn17-eNOS enabled full Akt-dependent activation of eNOS as determined by NO release and phosphorylation of Ser^{1179} to levels seen with WT eNOS. Plasma membrane-targeted eNOS constructs (C*AAX*-eNOS and Syn25-eNOS) exhibited constitutive NO release and phosphorylation of Ser^{1179}. In contrast to WT eNOS and Syn17-eNOS, cotransfection with Akt did not stimulate further production of NO or influence the phosphorylation state of Ser^{1179}.

Phosphorylation of Ser^{1179} Is Required for the Activation of Golgi Complex- and Plasma Membrane-restricted eNOS—The ability of Akt to increase eNOS activity is dependent on the specific phosphorylation of Ser^{1179}, and mutation of this site to the phospho-mimetic aspartic acid is sufficient to increase NO release from eNOS in the absence of additional stimuli (7, 14, 20). Therefore, we next investigated whether the phosphorylation state of this site is both sufficient to regulate NO release from cytosolic G2A eNOS and Golgi complex-targeted GRIP-eNOS and necessary for the enhanced activity seen with plasma membrane-targeted eNOS. As shown in Fig. 5*A*, mutation of Ser^{1179} to the phospho-mimetic aspartic acid (S1179D) increased NO release from the WT enzyme as described previously (7, 14). NO release from cytosolic G2A eNOS and GRIP-eNOS was also increased by the S1179D mutation, restoring the levels of NO release back to the WT enzyme levels. In contrast, the Syn17-S1179D construct was fully activated by the aspartate substitution and produced as much NO as WT eNOS transfected with Akt (compare the *second* and *eighth lanes*).

Next, we examined the importance of phosphorylation of Ser^{1179} in plasma membrane-targeted constructs that are constitutively active and hyperphosphorylated at Ser^{1179}. Mutation of Ser^{1179} to alanine (WT S1179A), which prevents phosphorylation, significantly reduced NO production from the WT enzyme as well as from the plasma membrane-targeted forms of eNOS (Syn25-eNOS and C*AAX*-eNOS), which constitutively produced more NO than the WT enzyme (Fig. 5*B*). There was no significant difference in the levels of NO produced from WT S1179A, C*AAX*-S1179A, and Syn25-S1179A. Thus, phosphorylation of eNOS at Ser^{1179} restricted to either the *cis*/medial-Golgi network or the plasma membrane primarily controls NO release.

DISCUSSION

It is well established that eNOS is localized to both the plasma membrane and the cytoplasmic aspects of the *cis*/medial-Golgi complex in endothelial cells both in intact blood vessels and in culture (25, 27, 28, 30, 33, 42–45). More recently, transgenesis of the entire human eNOS gene encoding an eNOS-green fluorescent protein recapitulated imaging and functional studies of eNOS-green fluorescent protein in cultured cells (43) and unequivocally documented the bimodal distribution of eNOS into the Golgi complex and plasma membrane of endothelial cells lining intact blood vessels (46). However, the presence of eNOS in both locations has complicated the process of identifying the individual roles played by each pool of eNOS and has made it difficult to determine whether the Golgi pool is merely a biosynthetic precursor pool of enzyme in route to the plasma membrane or an active pool of enzyme.

In this study, we generated fusion proteins that specifically target the plasma membrane or cytoplasmic aspects of the Golgi complex. All constructs were expressed well in cells and exhibited ample NOS activity *in vitro*. Surprisingly, plasma membrane-targeted eNOS (Syn25-eNOS and C*AAX*-eNOS) had slightly lower catalytic activity *in vitro*, but was constitutively active and phosphorylated at Ser^{1179} in intact cells and highly responsive to transmembrane calcium fluxes, yet insensitive to further activation by Akt-mediated phosphorylation. Mutation of Ser^{1179} to alanine prevented phosphorylation of this site and abolished the "constitutive activation" of eNOS brought about by targeting the plasma membrane. In contrast, *cis*-Golgi complex-targeted eNOS (Syn17-eNOS) behaved similarly to WT eNOS, was less sensitive to calcium-dependent activation, and was highly responsive to Akt-dependent phosphorylation compared with plasma membrane-targeted versions. We have shown that discrete targeting to the *cis*/medial-Golgi complex (Syn17-eNOS), but not to the *trans*- and post-Golgi vesicles (GRIP-eNOS), efficiently regulated eNOS

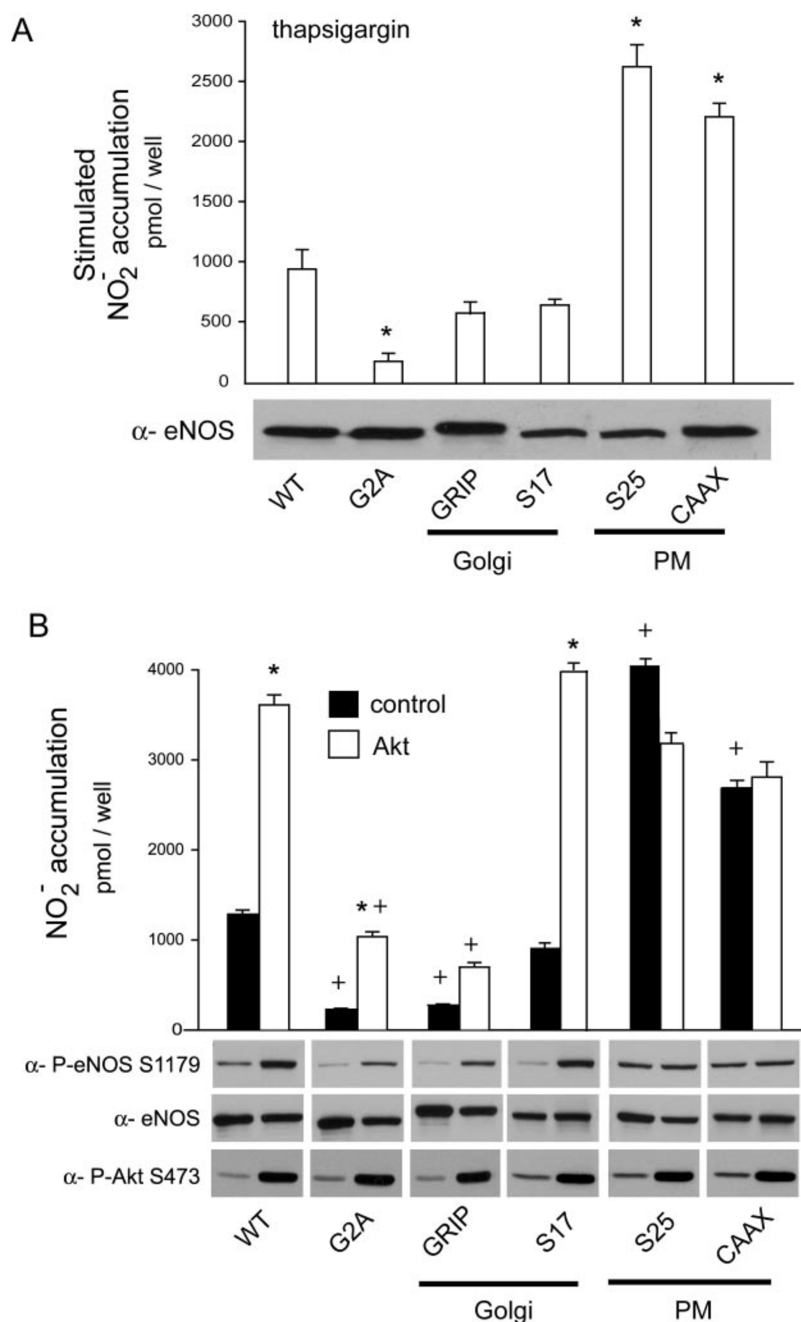

FIG. 4. **Calcium- and Akt-dependent activation of plasma membrane-targeted eNOS fusion proteins is greater than that of WT and Golgi complex-targeted eNOS constructs.** *A*, transfected COS cells expressing WT eNOS, G2A eNOS, or Golgi complex-targeted (GRIP-eNOS and Syn17-eNOS (*S17*)) or plasma membrane (*PM*)-targeted (Syn25-eNOS (*S25*) and C*AAX*-eNOS) eNOS constructs were stimulated with thapsigargin (1 μM) for 30 min, and stimulated NO release was determined by NO-specific chemiluminescence. Stimulated NO release was determined as the increase in NO release following subtraction of background levels. Data are presented as means ± S.E. ($n = 5$). *, $p < 0.05$ *versus* the WT enzyme. *B*, COS cells were transfected with the various eNOS constructs with or without the protein kinase Akt, and NO release was determined over a 24 h-period. Lysates were Western-blotted for the relative degree of Ser^{1179} phosphorylation (*upper gels*) relative to total eNOS (*middle gels*) and phosphorylated Akt (*lower gels*). Data are presented as means ± S.E. ($n = 5$). +, $p < 0.05$ *versus* the WT enzyme; *, $p < 0.05$ in the presence of Akt.

activation by Akt and NO release. The introduction of a negative charge at Ser^{1179} (S1179D) was sufficient to fully stimulate NO production from WT eNOS and Syn17-eNOS, consistent with their constitutive phosphorylation brought about by plasma membrane targeting. This study provides the first clear evidence for functional roles of plasma membrane- and Golgi complex-localized eNOS and demonstrates the paramount importance of Ser^{1179} phosphorylation in governing eNOS function in these specific locales in intact cells.

The central dogma of eNOS activation describes a model in which eNOS must be localized to plasma membrane microdomains such as caveolae or lipid rafts to produce optimal amounts of NO. This stems from several lines of evidence. Myristoylation- and/or palmitoylation-deficient mutants of eNOS fail to target both the plasma membrane and Golgi complex and display reduced activity *in vivo* (25, 26, 45). The majority of cellular eNOS and eNOS activity, normalized to caveola protein content, is concentrated in caveolin-1-enriched microdomains (25). Confluent endothelial cells have more plasma membrane and less Golgi eNOS than do subconfluent cells and release more NO in response to agonist challenge (42). The eNOS-binding proteins NOSIP and NOSTRIN displace plasma membrane eNOS to internal membranes (unlikely Golgi) and reduce calcium-dependent NO release (47, 48). And finally, depletion of plasma membrane cholesterol with β-methyl cyclodextrin and treatment with oxidized low density lipoprotein reduce plasma membrane eNOS to an intracellular compartment (non-Golgi) and attenuate NO release (49, 50). The majority of these studies have investigated the impact of subcellular targeting on the calcium-dependent activation of eNOS. Indeed, our data support the concept that targeting eNOS to the plasma membrane with either the full-length transmembrane domain of syntaxin-3 (Syn25-eNOS) or the prenylated polybasic domain of K-Ras (C*AAX*-eNOS) results in enzymes that are constitutively active and highly responsive to the calcium-mobilizing agent thapsigargin. However, in addition to calcium, eNOS can be regulated via phosphorylation of Ser^{1179}

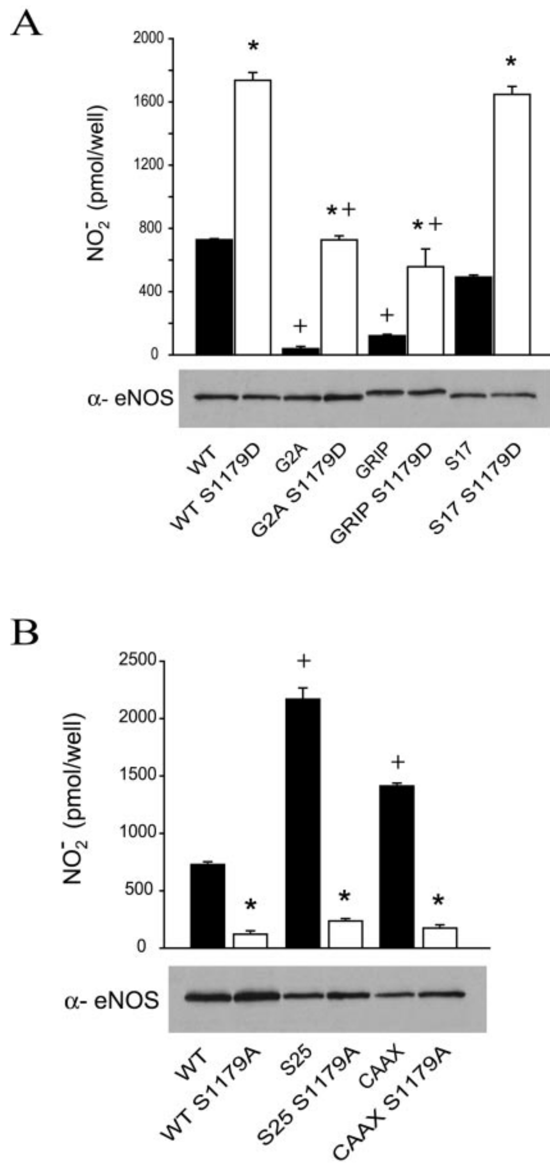

FIG. 5. **Phosphorylation of eNOS at Ser[1179] is both necessary for the constitutive activation of plasma membrane-targeted eNOS and sufficient to increase NO release from Golgi complex-targeted eNOS.** COS cells were transfected with WT eNOS, G2A eNOS, Golgi complex-targeted (GRIP-eNOS and Syn17-eNOS) and plasma membrane-targeted (Syn25-eNOS and C*AAX*-eNOS) eNOS constructs, and site-directed mutants of each construct in which Ser[1179] was mutated to alanine to prevent phosphorylation (WT eNOS, Syn25-eNOS, and C*AAX*-eNOS) or to the phospho-mimetic aspartic acid (WT eNOS, G2A eNOS, GRIP-eNOS, and Syn17-eNOS). *A*, mutation of Ser[1179] to aspartic acid increased NO release from WT eNOS, G2A eNOS, and Golgi complex-restricted eNOS constructs (GRIP-eNOS and Syn17-eNOS). *B*, mutation of Ser[1179] to alanine, which prevents phosphorylation, reduced NO release from WT eNOS and plasma membrane-targeted eNOS constructs (Syn25-eNOS and C*AAX*-eNOS). Data are presented as means ± S.E. (n = 5). *, $p < 0.05$ *versus* the corresponding unmutated enzyme; +, $p < 0.05$ *versus* WT eNOS.

by several protein kinases, including Akt. Thus, the relative contribution of subcellular targeting to agonists that utilize calcium- *versus* Akt-dependent mechanisms to activate eNOS remains to be established despite the evidence provided above.

In the majority of transfected cells, cultured endothelial cells, and intact blood vessels, a significant proportion of eNOS is found localized to the Golgi complex, where it co-localizes with Golgi markers, including mannosidase II and 58-kDa Golgi protein (27, 33, 51) and GM130 (this study). The function of Golgi complex-localized eNOS is poorly characterized, and given the preeminence placed on plasma membrane targeting, it is often assumed to be an inactive reservoir or immature eNOS in route to the plasmalemma and has more recently been interpreted as eNOS bound to internalized caveolae (51, 52). However, there is ample evidence for a functional role of Golgi complex-localized eNOS. As originally described in transfected cells (33, 37), eNOS is found in the peripheral aspects of the Golgi complex, and this targeting is required for optimal NO release from cells. More recently, using immunofluorescence microscopy to label eNOS phosphorylated at Ser[1179] and NO imaging with 4,5-diaminofluorescein diacetate, we found that, in response to the angiogenic vascular endothelial growth factor, Golgi complex-localized eNOS is phosphorylated at Ser[1179], and we identified "bursts" of NO emanating from the perinuclear region of endothelial cells (30). Recently, using a chimeric eNOS fusion protein fused to a calcium-sensing fluorophore, Jobin *et al.* (52) showed that calcium activation of eNOS imaged in living cells is primarily localized in a perinuclear orientation, identical to the localization of Golgi markers. In addition, acute chemical disruption of the Golgi complex inhibits NO release in some (53), but not all (54), endothelial cells.

In this study, we used a more direct approach to target eNOS specifically to the Golgi complex. Targeting eNOS to the *trans*- and post-Golgi vesicles via the GRIP domain resulted in an enzyme that produced very low amounts of basal NO and that was hyporesponsive to both thapsigargin and coexpression of Akt compared with the WT enzyme. The GRIP domain comprises ~42 amino acids and is shared by a family of resident peripheral Golgi coiled-coil membrane proteins, including p230, golgin-97, and golgin-245 (34). The GRIP docking site has been identified on a specific subset of Golgi membranes in the *trans*-Golgi network (55). Targeting eNOS to the *cis*/medial-Golgi complex (35) with the truncated transmembrane domain of syntaxin-3 (Syn17-eNOS) produced contrasting results. Compared with the WT enzyme, the calcium-dependent activation of Syn17-eNOS was slightly attenuated, but interestingly, this construct was strongly activated by Akt, supporting the idea that even within distinct membrane domains of the Golgi complex, compartmentalization is critical for eNOS activation. In other experiments, we attempted to place eNOS in the lumen of the Golgi complex via fusion with β-galactosyltransferase, but this construct did not express well, and the protein was degraded (data not shown). It is possible that targeting to the cytoplasmic aspect of the *trans*- *versus cis*-Golgi complex places the enzyme in a more favorable setting to produce NO or to be more accessible to kinases that can influence its activity. To address this question, we constructed phospho-mimetic mutants (S1179D) of GRIP-eNOS and Syn17-eNOS. The introduction of a negative charge at Ser[1179] resulted in increased NO release from GRIP-S1179D; however, its activity was increased only to the level of WT eNOS and was substantially less compared with WT S1179D and Syn17-S1179D. Thus, it is likely that factors other than phosphorylation of Ser[1179] contribute to the reduced activity seen in the GRIP-eNOS construct.

Subcellular targeting is known to influence both eNOS activity and phosphorylation (30, 31). In this study, we found that plasma membrane-targeted eNOS (*i.e.* C*AAX*-eNOS and Syn25-eNOS) was constitutively phosphorylated at Ser[1179] and that mutation of Ser[1179] to alanine abolished constitutive activation and reduced their activity to that of S1179A eNOS. Golgi complex-targeted Syn17-eNOS exhibited lower basal phosphorylation *versus* the WT enzyme, but was strongly phosphorylated in the presence of Akt, consistent with the ability of Akt to stimulate NO release. In contrast, *trans*-Golgi complex-targeted GRIP-eNOS displayed reduced basal and Akt-stimulated

phosphorylation comparable with cytosolic G2A eNOS. In addition to Ser[1179], eNOS is phosphorylated at Ser[116], Thr[497], Ser[616], and Ser[635], and all of these sites have been proposed to influence eNOS function. Given the importance of location to the specificity of kinases, it is perhaps not surprising that phosphorylation of eNOS at the other sites varied depending on where the enzyme was targeted. Phosphorylation of Thr[497], previously characterized as a negative regulatory site (16, 56) or more recently as a site important for the fidelity of eNOS coupling (18), was elevated in WT and plasma membrane-targeted eNOS. The phosphorylation of Ser[617] followed a similar pattern in that it was elevated in WT and plasma membrane-targeted eNOS, whereas *trans*-Golgi complex-targeted GRIP-eNOS had the most prominent phosphorylation of Ser[635]. The degree of phosphorylation of Thr[497], Ser[617], and Ser[635] was in general lower for *cis*-Golgi complex-targeted Syn17-eNOS. The functional significance of the differences in relative phosphorylation of these sites is not well understood compared with the better characterized Ser[1179] phosphorylation site.

The heterogeneous localization of eNOS is known to vary depending on the species, degree of confluency, passage, and even across the vascular tree (27, 28, 42, 43). The mechanisms regulating the intracellular distribution of eNOS between the Golgi and plasma membrane pools and the physiological role underlying their distinct regulation remain to be established. However, it is conceivable that by influencing the relative proportion of eNOS in the Golgi complex *versus* the plasma membrane, the ability of an endothelial cell to respond to calcium- and Akt-dependent agonists can be modified. For example, endothelial cell agonists that utilize primarily Akt-dependent mechanisms, *viz.* vascular endothelial growth factor, insulin, and estrogen, would preferentially active Golgi eNOS, and conversely, calcium-dependent agonists such as thapsigargin and bradykinin would preferentially activate plasma membrane eNOS. This concept is strengthened by the recent discoveries of two novel eNOS-binding partners, NOSIP and NOSTRIN (47, 48), and an indirect regulator, CHIP (57). Overexpression of both NOSIP and NOSTRIN influences eNOS activity only *in vivo* by redistributing eNOS from the plasma membrane to intracellular sites. CHIP, the C-terminal interacting protein for hsp70, is part of the eNOS-hsp90-hsp70 complex and, when overexpressed in endothelial cells, results in eNOS insolubility in the Golgi complex and loss of NO release from cells. Another consideration is the metabolic fate of locally generated NO. NO is typically thought to react with thiol residues of effector proteins in a diffusion-limited reaction; however, recent data showing that the greatest amount of NO is in the perinuclear region of cells and localized *S*-nitrosylation reactions influencing vesicle secretion challenge this dogma (58, 59). NO generated in the perinuclear/Golgi area would be more highly concentrated in membranes of the Golgi complex and around the nucleus and more apt to produce autocrine actions than NO synthesized in the plasma membrane, which may readily diffuse to extracellular targets. The autocrine role for NO in regulating endothelial cell function is supported by many studies in cultured endothelial cells showing that blockage of NOS reduces growth-stimulated cell migration, proliferation, and organization into tube-like structures (60–62) and a recent study in which NO was shown to inhibit von Willebrand factor secretion from Weibel-Palade bodies via nitrosylation of the protein NSF (*N*-ethylmaleimide-sensitive factor) (59). Whether the localized release of NO differentially contributes to autocrine (endothelial) or paracrine (vascular smooth muscle, etc.) actions of eNOS-derived NO remains to be established.

In summary, we have shown that plasma membrane- and Golgi complex-localized eNOS can indeed function to produce NO, exhibiting attributes similar to those of the WT enzyme. We have also shown that phosphorylation of Ser[1179] is of paramount importance for activation of plasma membrane- and Golgi complex-targeted eNOS to produce biologically active NO, regardless of the mechanism of activation of these pools of enzyme (*i.e.* calcium-dependent agonist *versus* Akt). Finally, our data strongly support that the concept of endomembrane-specific regulation of signaling systems, previously thought to exist only in the plasma membrane, is indeed operational for eNOS as highlighted recently by several classes of signaling molecules, including phosphatidylinositol 3,4,5-trisphosphate (63), Ras (64), and G proteins (65), now appreciated to signal on the Golgi and internalized membranes.

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