

The Amino-Terminus of Nitric Oxide Sensitive Guanylyl Cyclase α_1 Does Not Affect Dimerization but Influences Subcellular Localization

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Abstract

Background: Nitric oxide sensitive guanylyl cyclase (NOsGC) is a heterodimeric enzyme formed by an α - and a β_1 -subunit. A splice variant (C- α_1) of the α_1 -subunit, lacking at least the first 236 amino acids has been described by Sharina et al. 2008 and has been shown to be expressed in differentiating human embryonic cells. Wagner et al. 2005 have shown that the amino acids 61–128 of the α_1 -subunit are mandatory for quantitative heterodimerization implying that the C- α_1 -splice variant should lose its capacity to dimerize quantitatively.

Methodology/Principal Findings: In the current study we demonstrate preserved quantitative dimerization of the C- α_1 -splice by co-purification with the β_1 -subunit. In addition we used fluorescence resonance energy transfer (FRET) based on fluorescence lifetime imaging (FLIM) using fusion proteins of the β_1 -subunit and the α_1 -subunit or the C- α_1 variant with ECFP or EYFP. Analysis of the respective combinations in HEK-293 cells showed that the fluorescence lifetime was significantly shorter (≈ 0.3 ns) for α_1/β_1 and C- α_1/β_1 than the negative control. In addition we show that lack of the amino-terminus in the α_1 splice variant directs it to a more oxidized subcellular compartment.

Conclusions/Significance: We conclude that the amino-terminus of the α_1 -subunit is dispensable for dimerization *in-vivo* and *ex-vivo*, but influences the subcellular trafficking.

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Introduction

Nitric oxide sensitive guanylyl cyclase is the physiological receptor for nitric oxide (NO) and nitric oxide releasing drugs. Its second messenger cyclic GMP is crucial for vasodilatation, penile erection, platelet disaggregation and neurotransmission. The heterodimeric enzyme is formed by either an α_1 - or an α_2 -subunit and a β_1 -subunit. Dimerization of the enzyme is a prerequisite for its catalytic activity, because both the α - as well as the β_1 -subunit provide essential residues for the conversion of GTP to cGMP [1]. There is conflicting evidence which parts of the subunits are mandatory for quantitative heterodimerization. Previous studies [2,3] show inconsistent results with respect to the requirement of the α_1 subunit amino-terminus which is missing in the naturally occurring C- α_1 splice variant [4]. Since the occurrence of this splice variant has been linked to differentiation of human embryonic stem cells [5], the capacity of the C- α_1 splice variant to heterodimerize and thus form a functionally active enzyme, is of biological importance.

Because of the controversial nature of the question, we investigated the dimerization capacity using two independent experimental approaches. First we used a purification method of the NOsGC β_1 -subunit and looked for co-purification of the α_1 variants. With the second experimental approach we examined fluorescence resonance energy transfer (FRET) based on fluorescence lifetime imaging (FLIM) in intact cells using fusion proteins of the respective subunits with fluorescent proteins. While analyzing the fluorescence lifetimes of the respective NOsGC-subunits, we serendipitously discovered that the C- α_1 splice isoform, shows a unique subcellular distribution. Using a fluorescent tagged marker for the endoplasmic reticulum (ER), we conclude, that the C- α_1 isoform is located at the ER. As it is well known, that the redox state of the ER is relatively more oxidized than the cytosol [6], we performed a ratiometric analysis of the redox properties of the C- α_1 protein by using a redox sensor (Grx1-roGFP2) and identified, that the protein is not only distributed in a different manner than the wild type, but also located in a more oxidized environment. Co-expression of the β_1 -

subunit restored the cytosolic localization of the C- α_1 splice isoform, but led to a nuclear localization that was not found for the canonical α_1/β_1 heterodimer. We could show that the C- α_1 splice isoform retains its ability to heterodimerize quantitatively with the β_1 -subunit *ex vivo* and in intact cells, despite lack of amino-terminal amino acids that were thought to be important for heterodimerization [2]. In addition, we observed that the fluorescent fusion of the C- α_1 splice subunit is directed to a more oxidized subcellular compartment, while the respective C- α_1/β_1 heterodimer shows a cytosolic and nuclear localization.

Results

$\alpha_1\Delta N_{259}$ will be expressed by the C- α_1 splice form

Analyzing the C- α_1 splice-variant described by Sharina et al. [4] and designated $\alpha_1\Delta N_{240}$ shows that the first initiation codon after splicing would either form an $\alpha_1\Delta N_{236}$ or an $\alpha_1\Delta N_{259}$ variant because these represent the only methionines with an open reading frame in the human sequence (Fig. 1 and Data S2). In a recently published review by Sharina et al. 2011, the authors have adapted our numbering [7]. In a previous study by Koglin and Behrends 2003 [3], we investigated intensely the $\alpha_1\Delta N_{236}$ truncation in comparison to the $\alpha_1\Delta N_{259}$ truncation. Because neither enzyme activity, substrate-dependency (GTP), nor dose-effect-curve of nitric oxide differed significantly from $\alpha_1\Delta N_{259}$, it was suggested by the reviewers to remove the redundant data for the $\alpha_1\Delta N_{236}$ truncation. Since there was no difference in molecular weight between the $\alpha_1\Delta N_{236}$ and $\alpha_1\Delta N_{259}$ variant (Fig. 2), we now assume that the recognition of the translation initiation site of ATG₂₅₉ is dominant over ATG₂₃₆ in the human sequence. We thus suggest that the C- α_1 splice variant leads to the formation of a subunit with an $\alpha_1\Delta N_{259}$ deletion.

Establishment of a novel one-step-purification protocol

We performed a new one-step-purification of the soluble guanylyl cyclase using the *Strep* Tag II, which results in 1 mg purified NOsGC from 1,000 ml *Sf*-9 culture medium. The yield calculated by using the total activities of cytosol and purified protein is $\approx 25\%$. The purification factor is ≈ 125 -fold and results in a more than 95% purity of the enzyme determined by Coomassie stained gels.

Analysis of the heterodimerization of C- α_1 by co-purification with β_1 -S

Using a carboxy-terminally *Strep* tagged β_1 -subunit of NOsGC, we could show that both α_1 -isoforms dimerize with the β_1 -subunit

(Fig. 3). Densitometric analysis and graphical representation as described in [2] shows that C- α_1 forms heterodimers not less but more effectively with a relative value of 166.2% ($\pm 12.2\%$) compared with α_1 WT (Fig. 4). Alternatively the ratio of the band intensities of the α_1 WT variants to the β_1 -bands were 0.75 ± 0.11 for C- α_1/β_1 and 0.43 ± 0.06 for α_1/β_1 . The excess of the β_1 subunit is due to the fact that purification was performed with a *Strep* Tag II attached to the β_1 -subunit as described in [2]. Because our findings contradict the central message of the paper by Wagner et al. 2005 although the experimental approach was very similar [2], we tested more subtle differences. We expressed *Sf*-9 cells without any supplement, only with hemin (4 mg/l) and with both hemin and lipid supplement as in [2], the latter being used to reduce the shear forces during *Sf*-9 cell culture. After expression under these conditions, we purified the enzyme complex and performed a SDS-PAGE and the gel was stained with Coomassie Blue according to the protocol by Kang et al. [8]. There was no negative effect of either of the supplements on the heterodimerization of C- α_1/β_1 S (Fig. 5) confirming our finding that the C- α_1 splice-variant retains its ability to heterodimerize under different experimental conditions.

Guanylyl cyclase activity of the purified C- α_1/β_1 and α_1/β_1 heterodimers was measured under basal conditions, in the presence of NO (100 μ M DEA/NO) or in the presence of 10 μ M cinaciguat. Consistent with the formation of more functional heterodimers for C- α_1/β_1 versus α_1/β_1 in *Sf*-9 cell culture, enzyme activity was higher for the splice variant under all experimental conditions (Fig. 6). Spectroscopic analysis shows that both C- α_1/β_1 than α_1/β_1 contain a significant amount of heme (Fig. 7) which is consistent with their responsiveness to nitric oxide (see Fig. 6).

Analysis of the heterodimerization of C- α_1 by FLIM-FRET

In order to test whether the unexpected formation of more functional heterodimers with the β_1 subunit for C- α_1 versus the canonical α_1 subunit is also seen in intact cells we used fluorescence resonance energy transfer (FRET) based on fluorescence lifetime imaging. The α_1 -subunit and C- α_1 -subunit were fused with ECFP and the β_1 -subunit with EYFP in analogy to a previous FRET study by our group [9]. Analysis of the respective combinations in HEK-293 cells showed that the fluorescence lifetime was significantly shorter for α_1/β_1 and C- α_1/β_1 than the negative control (Fig. 8A). This indicates that heterodimerization of both α_1 variants with the β_1 subunit bring the respective fused fluorescent proteins in a physical distance below 80 Å [10]. As an additional control, FLIM-FRET experiments were performed with

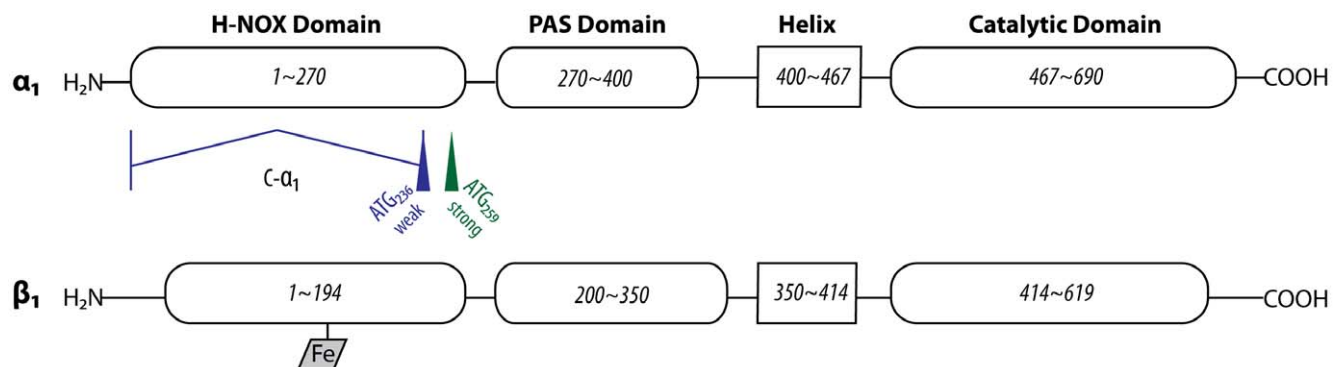


Figure 1. Assumed formation of $\alpha_1\Delta N_{259}$ deletion through C- α_1 splice variant of guanylyl cyclase. The graphic is a modified version of the domain architecture model by Derbyshire and Marletta [34]. doi:10.1371/journal.pone.0025772.g001

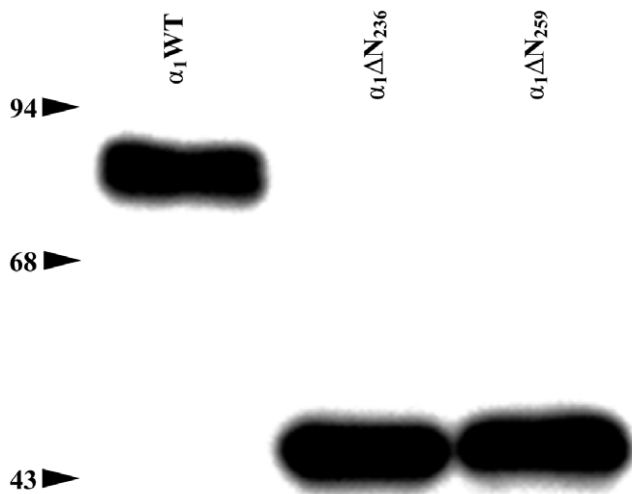


Figure 2. Characterization of expression of the α_1 -subunits by Western-blot analysis in cytosolic fractions of *Sf-9* cells infected with the respective variants of the α_1 -subunits. All lanes were loaded with 40 μ g protein of the cytosolic fractions. The experiments were repeated at least three times and one representative result is shown. [molecular weights predicted from their amino acid sequences: α_1 WT, 77.6 kDa; $\alpha_1\Delta N_{236}$, 51.2 kDa; $\alpha_1\Delta N_{259}$, 48 kDa]. doi:10.1371/journal.pone.0025772.g002

constructs where the fluorescent proteins were exchanged (Fig. 8B). Again there was evidence that the C- α_1 splice variant and the canonical α_1 -subunit heterodimerize equally well in intact cells.

The results are expressed as means \pm SEM of at least three independent experiments. For minimum 20 cells were analyzed each. All results were controlled for their statistical significance by Student's t-test. A value of $p < 0.01$ was considered to be statistically significant.

The magnitude of the change of the fluorescence-lifetime (≈ 0.3 ns) was expected to prove an interaction.

Subcellular distribution of C- α_1

While performing expression controls for the FLIM-FRET experiments, we noticed a different subcellular localization of the fluorescent fusion proteins of the C- α_1 splice variant versus the canonical α_1 subunit each expressed in the absence of the β_1 -subunit. Because of the granular perinuclear appearance of the splice variant, we co-expressed human heme oxygenase 1 (HO-1) with an amino-terminal ECFP as a well-known marker for the endoplasmic reticulum [11] (Fig. 9A and B). While the C- α_1 splice variant showed an exact co-localization with HO-1, the canonical α_1 subunit showed a diverging homogenous distribution in the cytosol (see Fig. 9A and B). Co-expression of untagged β_1 -subunit led to a homogenous wild type like distribution of the C- α_1 splice variant with an additional nuclear signal in some (Fig. 9C, red arrow) but not all cells (Fig. 9C, white arrow). Co-expression of CFP-tagged β_1 -subunit (and omission of CFP-HO1) demonstrates that expression of substantial amounts of the β_1 -subunit induces this translocation to the nucleus (Fig. 9D, red arrow). In contrast, no nuclear expression was detected for the canonical α_1 subunit in the presence of β_1 (Fig. 9E).

Because *Sharin et al.* [5] have shown that the C- α_1 splice variant is "uniquely resistant to oxidative protein degradation" we analyzed the redox-state of both α_1 variants by fusion with the redox-sensor Grx1-roGFP2. Ratiometric analysis showed no difference between Grx1-roGFP2 (Fig. 10A) and α_1 -Grx1-roGFP2 (Fig. 10B). In contrast, C- α_1 -Grx1-roGFP2 showed a higher

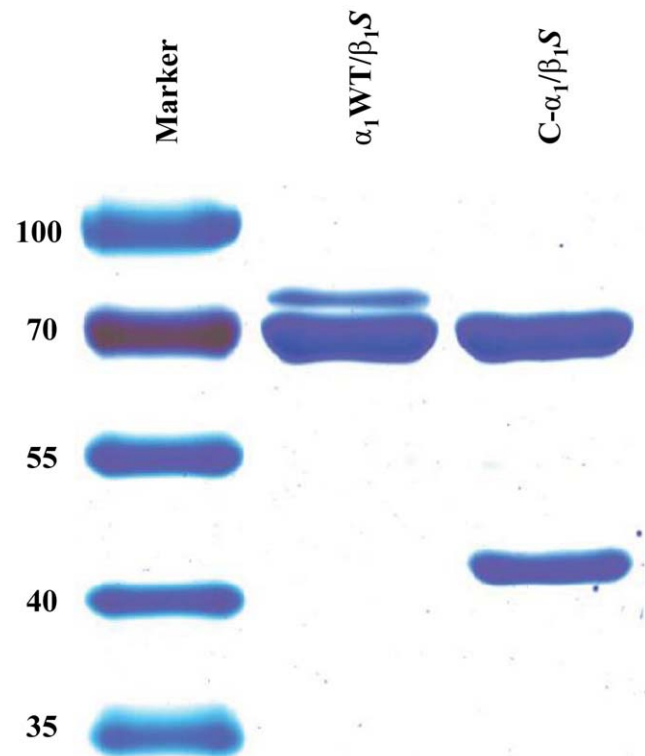


Figure 3. SDS-PAGE analysis of NOsGC variants. 5 μ g of each purified enzyme was electrophoretically separated by SDS-Page and stained with Coomassie Blue. The experiments were repeated at least three times and one representative result is shown. [molecular weights predicted from their amino acid sequences: α_1 WT, 77.6 kDa; C- α_1 , 48 kDa; β_1 S, 72.2 kDa]. doi:10.1371/journal.pone.0025772.g003

ratiometric signal indicative of a more oxidized subcellular environment (Fig. 10C). This effect was attenuated by co-expression of the untagged β_1 -subunit (Fig. 10D). In contrast, co-expression of untagged β_1 -subunit with α_1 -Grx1-roGFP2 led to no change (Fig. 10E in comparison to Fig. 10B).

Discussion

It has recently been shown that the α_1 -subunit of the nitric oxide receptor NOsGC undergoes splicing regulation in differentiating human embryonic cells [5]. The results indicated high levels of an amino-terminally deleted C- α_1 -splice form in differentiating cells that showed a different intracellular distribution in comparison to the canonical full-length α_1 -subunit. It has been published that the amino-terminus of the α_1 -subunit is important for quantitative dimerization with the β_1 -subunit [2]. This was in contrast to a study where we had shown co-purification of the β_1 -subunit with $\alpha_1\Delta N_{259}$ [3]. To explain the discrepancy, *Wagner et al.* [2] raised the argument that we had applied a purification method, where we pooled the fractions with catalytic activity [3]. Due to this approach, it was conceivable, that only a small fraction which has formed active heterodimers is considered, while non-heterodimerizing subunits are discarded. The present study circumvents this potential problem by using a purification procedure based on an affinity StrepTag attached to the β_1 -subunit in analogy to the study of *Wagner et al.* [2]. Our results obtained by using co-purification of the α_1 -subunit and the amino-terminally deleted C- α_1 -splice form with the StrepTagged β_1 -subunit indicate that the C- α_1 -splice form heterodimerizes quantitatively. Because of the

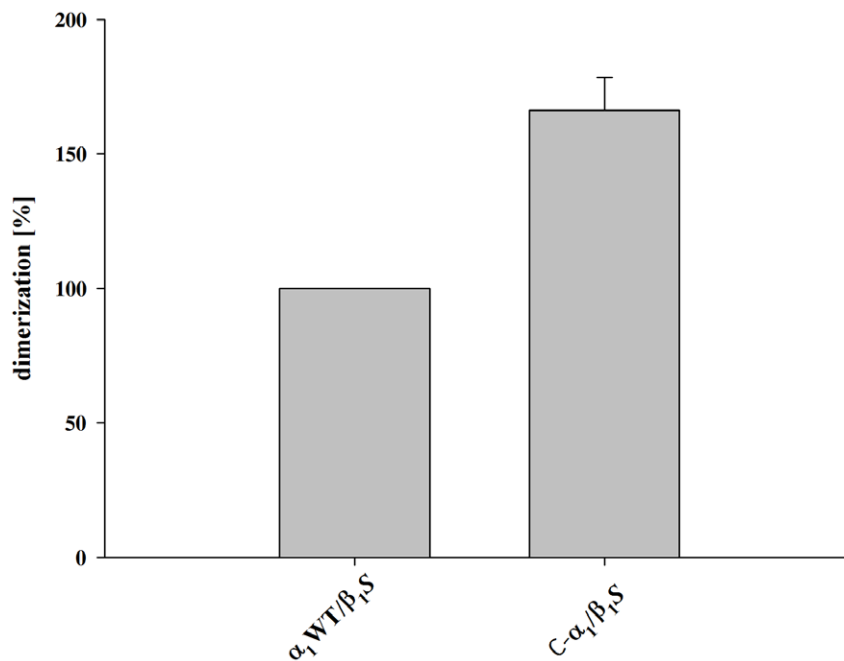


Figure 4. Densitometric analysis of dimerization. For quantification of the dimerization the optical densities of the Coomassie-stained bands were measured. A: The values obtained for α_1 were normalized to β_1 in the same lane. Dimerization was calculated in percentage of α_1 WT/ β_1 S. Data are expressed as means \pm SEM.
doi:10.1371/journal.pone.0025772.g004

controversial nature of the question, whether dimerization of nitric oxide sensitive guanylyl cyclase requires the α_1 amino-terminus, we used an additional method based on fluorescence resonance energy transfer to demonstrate heterodimerization of the C- α_1 -splice form with the β_1 -subunit in intact cells. This corroborated our finding that lack of the amino-terminus of the α_1 -subunit does not influence quantitative dimerization with the β_1 -subunit. This is also consistent with a number of other studies [4,12,13,14]. Our finding that α_1 -variants heterodimerize equally well in intact cells, but the C- α_1 splice form seems to lead to more intact heterodimers upon purification is probably due to the greater stability of the

C- α_1 splice variant [5]. This may also be the reason for the higher enzyme activity of the C- α_1 / β_1 heterodimer versus wild type. Alternatively, it is conceivable, that the amino-terminal region absent in C- α_1 has a negative regulatory influence on basal activity. This would be in line with a proposed regulatory domain-scale mechanism of the amino-terminal domains of NOsGC [15] that are in proximity to the catalytic domain [9]. The splice variant would thus represent a disinhibited isoform under basal conditions.

Using fluorescent fusion proteins, we noticed a peculiar localization of the C- α_1 -splice form compared to the wild type. Comparison with fluorescent tagged heme oxygenase-1 which is

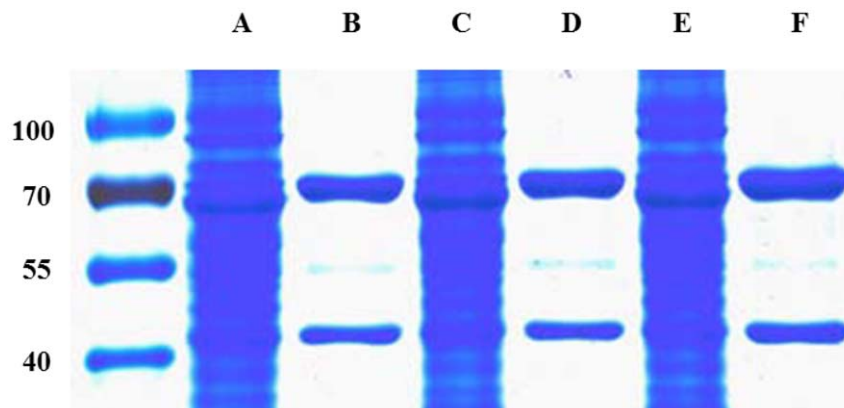


Figure 5. Coomassie staining of cytosolic fractions and purified proteins. 50 μ g Cytosol of cytosolic fraction or 5 μ g of purified enzyme was electrophoretically separated by SDS-PAGE and stained with Coomassie Blue (a representative gel is shown). Lane A, cytosolic fraction of C- α_1 / β_1 S expressed without heme-supplement and without lipid medium supplement. Lane B, purified C- α_1 / β_1 S expressed without heme-supplement and without lipid medium supplement. Lane C, cytosolic fraction of C- α_1 / β_1 S expressed only with heme-supplement and without lipid medium supplement. Lane D, purified C- α_1 / β_1 S expressed with heme-supplement and without lipid medium supplement. Lane E, cytosolic fraction of C- α_1 / β_1 S expressed only with heme-supplement and with lipid medium supplement. Lane F, purified C- α_1 / β_1 S expressed with heme-supplement and with lipid medium supplement.
doi:10.1371/journal.pone.0025772.g005

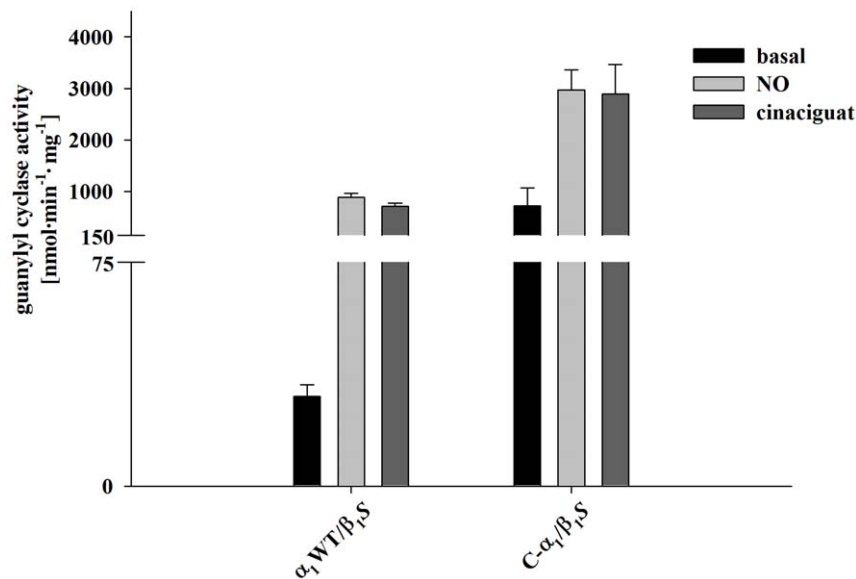


Figure 6. Guanylyl cyclase activity of purified variants of the enzyme. Specific activity was measured under basal conditions (black column), in the presence of 100 μ M NO (DEA/NO, white column) and in the presence of 10 μ M cinaciguat (gray columns). Data are expressed as means \pm SEM. The experiments were repeated at least three times and one representative result is shown. doi:10.1371/journal.pone.0025772.g006

known to be attached to the endoplasmic reticulum outer membrane [16], revealed a highly similar subcellular distribution. As the endoplasmic reticulum shows a relatively oxidizing thiol-disulfide milieu [17], we employed a novel method to measure the glutathione redox potential based on a fusion of the α_1 -subunit in its different splice forms with glutaredoxin-1 and roGFP2 [18]. This confirmed a more oxidizing thiol-disulfide milieu for the C- α_1 splice variant compatible with localization at the endoplasmic reticulum. Co-expression of the non-tagged β_1 -subunit changed the subcellular localization as well as the thiol-disulfide milieu signal for the C- α_1 -splice form but had no effect on the α_1 -subunit. The finding that the α_1 / β_1 -heterodimer showed the expected diffuse cytosolic localization, while the C- α_1 / β_1 -heterodimer was also present in the nucleus, is interesting in the context of reports suggesting a role for the β_1 -subunit in the nucleus: *Baltrons* and

colleagues have demonstrated that the β_1 -subunit translocates to the nucleus for proteosomal degradation in rat astrocytes after treatment with bacterial endotoxin [19]. In a subsequent report the β_1 -subunit was shown to be peripherally associated to chromosomes during mitosis and to play a role in chromatin condensation and cell cycle progression in rat C6 glioma cells [20]. The mechanism by which the β_1 -subunit enters the nucleus is unknown since the protein lacks a recognizable nuclear localization signal [19]. However, it is conceivable that regions of the β_1 -subunit that may be shielded in the classical α_1 / β_1 -heterodimeric enzyme are exposed in the C- α_1 / β_1 -heterodimer and the β_1 -homomer and are free to interact with proteins that regulate nuclear import [21].

Sharin et al. have analyzed the subcellular distribution of the native C- α_1 splice form in differentiating human embryonic cells

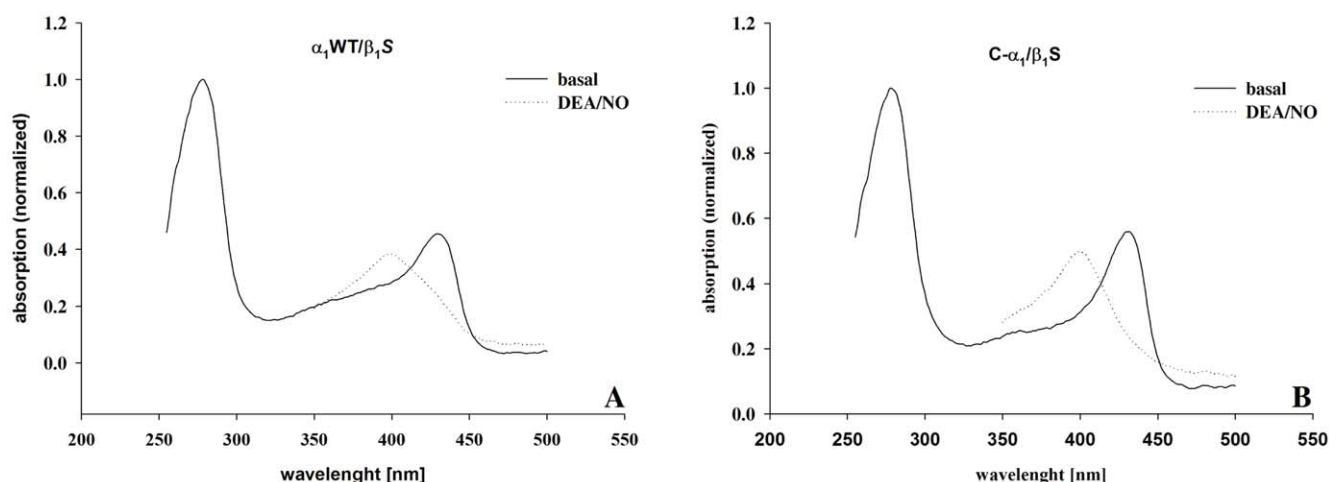


Figure 7. Spectroscopic analysis of purified cGC enzyme complexes. Spectroscopic analysis shows absorption values at basal (solid line) or NO-stimulated (100 μ M DEA/NO, dotted line) conditions. A: α_1 WT/ β_1 S. B: C- α_1 / β_1 S. doi:10.1371/journal.pone.0025772.g007

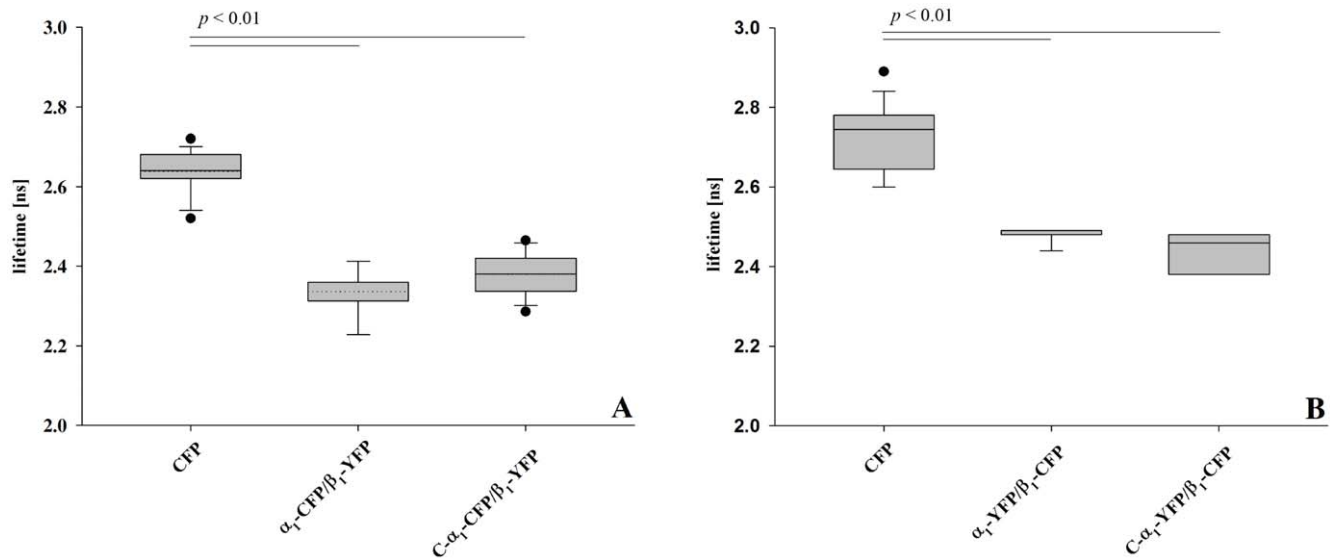


Figure 8. Fluorescence lifetimes of the FRET donor ECFP in HEK-293 cells, expressing donor only, and fluorescent tagged heterodimeric NOsGC variants. Mean fluorescence lifetimes of ECFP were measured in HEK-293 cells at 37°C, 48 h post transfection with the respective recombinant constructs. A: α_1 -variants as fluorescence-donor. B: α_1 -variants as fluorescence-acceptor. Data are presented in box and whiskers plots showing the 25th percentiles, 75th percentile and median as box with the mean value as dotted line. Whiskers represent the 5th and 95th percentile, dots are outliers. Fluorescent tagged NOsGC variants show significantly reduced fluorescent lifetimes due to FRET. doi:10.1371/journal.pone.0025772.g008

[5]: With biochemical methods the C- α_1 splice form could be detected in small amounts in the fraction containing nuclei and to a greater extent in the cytosolic fraction [5]. This is consistent with our findings for the overexpressed fluorescent tagged C- α_1 / β_1 -heterodimer. Immunocytochemical analysis of differentiating human embryonic cells at day 12 with an antibody that recognizes both the α_1 -subunit and the C- α_1 splice form showed a majority (88 %) of cells with a diffuse staining indicative of a predominant localization in the cytosol [5]. Again this is in line with our results for the fluorescent tagged C- α_1 / β_1 -heterodimer and the fluorescent tagged α_1 / β_1 -heterodimer. In a minority of cells (12 %), a filamentous staining was observed in human embryonic cells using an antibody that recognizes both the α_1 -subunit and the C- α_1 splice form. These cells were not analyzed for the β_1 -subunit expression. So it remains a possibility that these cells do express no or less β_1 -subunit. This would be consistent with our finding that the fluorescently tagged C- α_1 splice form shows a different subcellular localization and redox environment alone than in the presence of the β_1 -subunit.

The endoplasmic reticulum is involved in heme trafficking, heme degradation and heme insertion into hemoproteins in eukaryotic cells [22]. Biochemical analysis in native human embryonic cells showed small amounts of the canonical α_1 -subunit in the fraction containing the endoplasmic reticulum. The function of the H-NOX domain of the α -subunits that is absent in the C- α_1 splice variant is not clear. Very recently it has been suggested based on overexpression and purification of the H-NOX- α_1 -fragment in *E. coli* that it may bind heme via a non-covalent interaction [23]. It is possible that the lack of this domain in the C- α_1 protein leads to trapping of the splice variant at the site of heme insertion, while the canonical α_1 -subunit or the α_1 / β_1 -heterodimer interacts only transiently with the endoplasmic reticulum e.g. during heme insertion or maturation.

Sharina et al. showed that the C- α_1 splice variant is more stable in intact cells in the presence of ODQ, a well-known oxidant [24] while the canonical α_1 -subunit is more rapidly degraded

under these conditions. We show in the current paper that fluorescent labeled overexpressed C- α_1 protein is targeted to a more oxidized environment in comparison to the canonical α_1 -subunit. Thus the higher stability of the C- α_1 protein in the presence of oxidants may represent an adaption to the redox environment at the endoplasmic reticulum because it is well known to be a more oxidized compartment than the cytosol [6]. NOsGC activators, like cinaciguat, which act at the H-NOX domain of the β_1 -subunit enhance the stability of the protein through inhibition of proteosomal degradation which can be induced by oxidants like ODQ in the cell [25,26]. It is thus conceivable that heme or ligand free H-NOX domains not only of β_1 but also of α_1 subunits provide a signal that leads to more rapid degradation.

In summary, we present evidence that the amino-terminal H-NOX domain of the α_1 -subunit does not preclude quantitative dimerization with the β_1 -subunit to form a stable active, heme containing NOsGC-heterodimer but is important for subcellular localization.

Materials and Methods

Materials

Cinaciguat (BAY 58-2667) was a generous gift from *Johannes-Peter Stasch* (Bayer Pharma AG, Wuppertal, Germany). The *Sf-9* cells were obtained from Invitrogen (Karlsruhe, Germany), the HEK-293 cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) (Brunswick, Germany) and the *Sf-9* Easy Titer cell line (*Sf-9ET*) was a generous gift from *Dr. Dominic Esposito* (National Institutes of Health, Rockville, USA). D-desthiobiotin, avidin and *Strep-Tactin*[®] Superflow[®] high capacity resin were purchased from IBA, Goettingen, Germany. 2-diethyl-1-nitroso-oxyhydrazine (DEA/NO), 2-(4-Hydroxyphenylazo)benzoic acid (HABA), creatine kinase, hemin, lipid medium supplement and all other chemicals, in the highest grade of purity, were obtained from Sigma-Aldrich, Munich, Germany. [α -³²P]GTP (400 Ci/mmol) was purchased from Hartmann

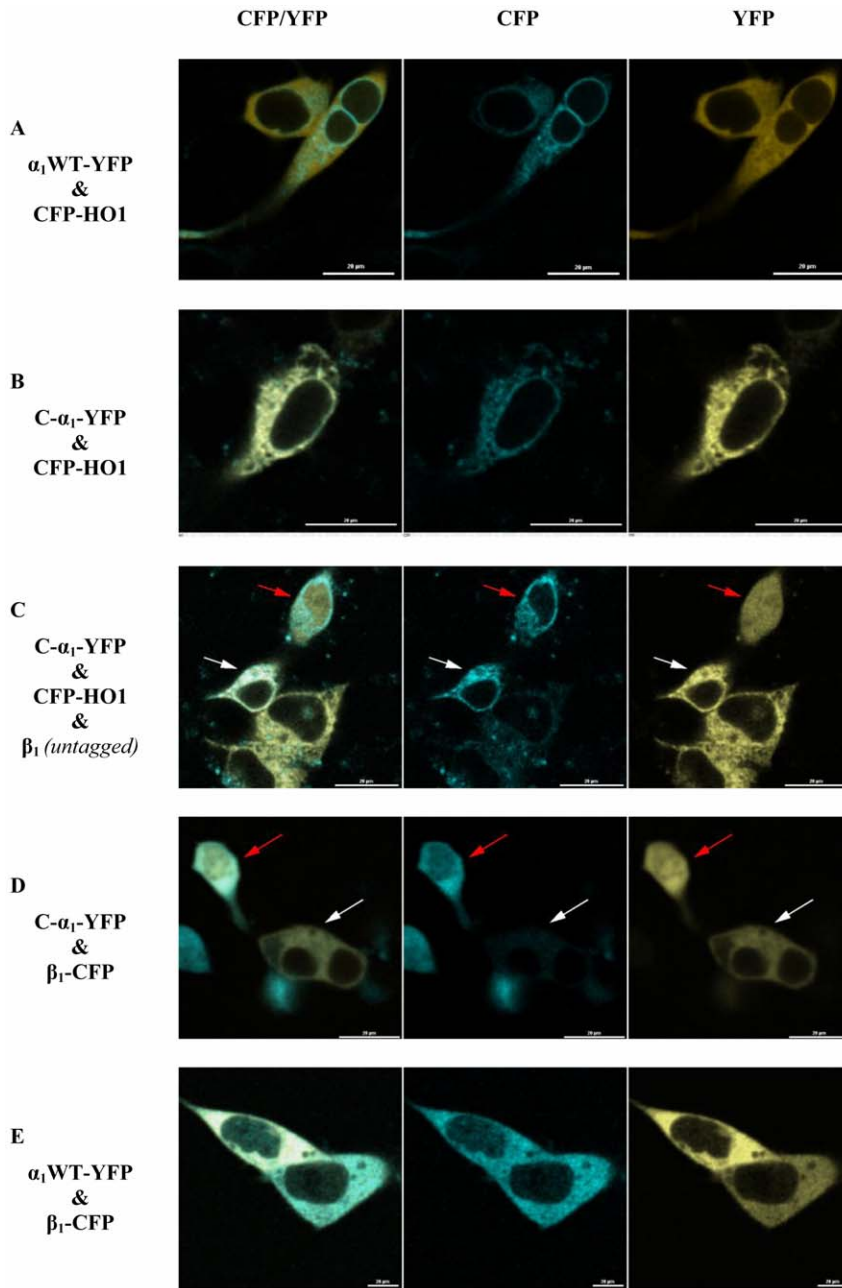


Figure 9. Expression of EYFP-marked α_1 full-length (A) and C- α_1 (B) in HEK-293 cells. As a marker of the endoplasmic reticulum [11] ECFP tagged heme oxygenase 1 (human, HO-1) was co-transfected. A, α_1 full-length shows cytosolic distribution. B, C- α_1 shows a similar distribution like ECFP-HO-1. C, addition of an untagged β_1 -subunit led in some cells a similar subcellular distribution as the wild type. The red arrow denotes a cell likely co-transfected with β_1 , the white arrow denotes a cell likely not co-transfected with β_1 . D, only cells which express both subunits show homogenous subcellular distribution. The red arrow denotes a cell co-transfected with β_1 , the white arrow denotes a cell not co-transfected with β_1 . E, The subcellular localization of α_1 full-length is not affected through coexpression of β_1 . Bar, 20 μm . CFP, ECFP channel; YFP, EYFP channel.
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Analytic, Brunswick, Germany. All primers used for site directed mutagenesis, were obtained in HPLC purity grade from biomers.net, Ulm, Germany.

Cloning of α_1 Deletion Mutants

For construction of the $\alpha_1\Delta\text{N}_{236}$ mutant, a *PvuII/HindIII* fragment of the α_1 full-length (see ref. [3]) clone was ligated *StuI/HindIII* into pFastBacTM1 vector (Invitrogen, Karlsruhe,

Germany). Cloning of the human amino-terminal deletion mutant $\alpha_1\Delta\text{N}_{259}$ has been described previously [3].

Cloning of carboxy-terminal Strep Tag II β_1 -subunit (Sf-9 system)

For construction of the carboxy-terminal *Strep* Tag II with the β_1 -subunit a *PvuI/StuI* fragment of the conjoined NOsGC construct $\beta_1\alpha_1$ -*Strep* [27] was cloned using *PvuI/SmaI* into the pFastBacTM1 vector.

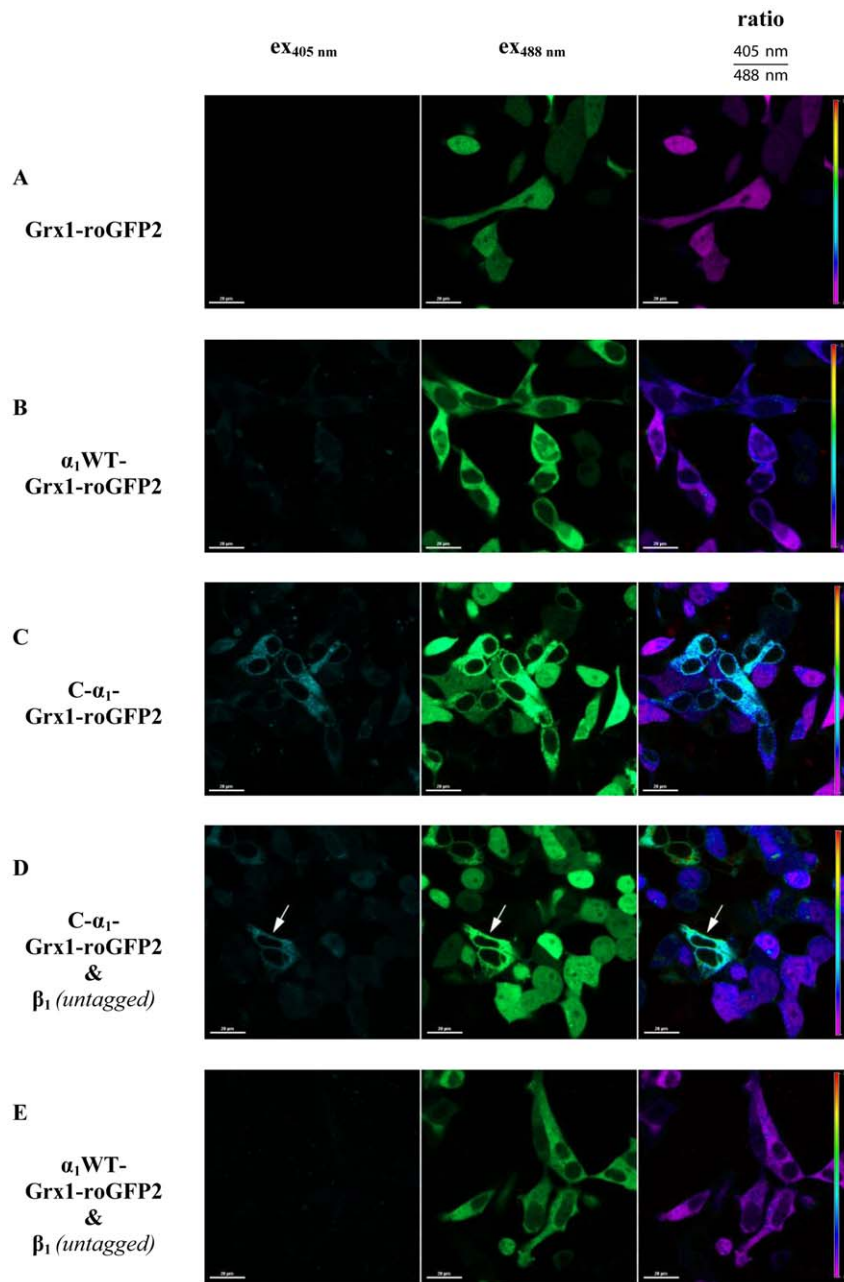


Figure 10. Redox-state analysis of the α_1 full-length and C- α_1 in HEK-293 cells, using the redox-sensor Grx1-roGFP2. According to the settings a higher value means a more oxidized state of the protein.
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Cloning of α_1 WT and C- α_1 fused with fluorescent proteins for determination of fluorescence lifetime

For construction of α_1 full-length in pECFP-N1 (Clontech, Mountain View, CA, USA) the α_1 cDNA in pcDNA3.1/V5/His-TOPO described by Haase et al. [27] was cloned *HindIII/XhoI* into *HindIII/SalI* pECFP-N1. Cloning of the C- α_1 (rat $\alpha_1\Delta N_{258}$) was done by introduction of suitable restriction sites by site directed mutagenesis into this construct, restriction and religation. At the same time the *Kozak* consensus sequence [28] was optimized and a start-methionine had to be introduced. For construction of C- α_1 -ECFP the following primer pair was used: 5'-GAA CCA GCC CTA TTT GCT CGA GTC GGT CGC CAT GGA GAG CAC

CAA GCC TTC TCT-3' and 5'-AGA GAA GGC TTG GTG CTC TCC ATG GCG ACC GAC TCG AGC AAA TAG GGC TGG TTC-3'. The initiation codons or complementary sequence are bold type and the modified nucleotides are underlined. Using the respective restriction sites in pECFP before the insert *XhoI* digestion and religation led to C- α_1 -ECFP. Due to a slight difference in the amino acid sequence rat $\alpha_1\Delta N_{258}$ correspond to human $\alpha_1\Delta N_{259}$ (Data S1).

Cloning of β_1 fused with EYFP for determination of fluorescence lifetime

The cloning has been described previously [27].

Exchange of ECFP and EYFP

The carboxy-terminal ECFP-fusions of α_1 full-length and C- α_1 were exchanged for EYFP and the carboxy-terminal EYFP-fusion of β_1 was exchanged for ECFP using the pEYFP-N1 or pECFP-N1 vector (Clontech) and *AgeI/BsrGI* as restriction enzymes.

Cloning of β_1 in pcDNA3.1/V5/His-TOPO for Expression in HEK-293-cells

The cloning has been described previously [27].

Cloning of ECFP-HO1 in pECFP-C1

The human heme oxygenase 1 (HO1) was amplified using the primer pair (5'-CCC AGC ACC GGC CGG ATG GAG-3'/5'-TTC AGT GCC CAC GGT AAG GAA GC-3') and the FirstChoiceTM PCR-Ready Human Placenta cDNA (Ambion, Austin, USA). The PCR product was subcloned into pCR[®]2.1-TOPO[®] vector. Through *EcoRI/XbaI* restriction the insertion into pFastBacTM1 was performed. Using *EcoRI/KpnI* the cDNA was transferred into pECFP-C1 [29].

Cloning of Grx1-roGFP2-tagged α_1 variants

Grx1-roGFP2 in pLPCX (Clontech) was a kind gift from *Dr. Tobias Dick* (DKFZ, Heidelberg, Germany) [18]. Through restriction with *BglIII/BsrGI* the construct was ligated into pEGFP-N1. Using *SmaI/XbaI* ECFP in α_1 -ECFP (s.a.) was exchanged for Grx1-roGFP2 out of pLCPX (*Eco47III/XbaI* restricted). After *ApaI* restriction and religation the frame was restored.

Restriction of C- α_1 -EYFP and α_1 -Grx1-roGFP2 with *Eco47III/BsrEII* led to an exchange of the full-length construct for the splice variant.

Control of sequences

All cloned constructs were verified by sequencing (GATC Biotech, Konstanz, Germany).

Baculovirus Generation

Recombinant baculoviruses of respective subunits were generated using the Bac-to-Bac[®] Baculovirus Expression System (Invitrogen).

Sf-9 Cell Culture, Expression of Recombinant Guanylyl Cyclase Subunits and Preparation of Cytosolic Fraction

Sf-9 cells were cultured in Sf-900TM II serum-free medium (Invitrogen) supplemented with 1 % penicillin/streptomycin (PAA Laboratories, Coelbe, Germany) and 10 % fetal bovine serum (Foetal Bovine Serum Gold, EU-approved, PAA Laboratories). Spinner cultures were grown at 27°C at 140 rpm shaking on a 50 mm orbit platform and diluted to $2 \cdot 10^6$ cells/ml for infection. 500 ml of cell solution were infected with the respective recombinant baculovirus stock with the multiplicities of infection (MOI) of 1. The MOI was determined using Sf-9ET cell line according to the recent published paper by *Hopkins and Esposito* [30]. After 72 h cells were harvested by centrifugation (4,000 g for 1 min at 4°C). All following steps were performed on ice. The cell pellet was resuspended in 30 ml of lysis buffer containing 50 mM TEA-HCl, 1 mM EDTA, 10 mM dithiothreitol (DTT), 250 nM Avidin, pH 7.4, and complete EDTA-free Protease Inhibitor Cocktail Tablets (Roche, Mannheim, Germany). The cells were lysed by sonication. Cytosolic fractions were obtained by centrifugation for 180 min at 15,000 g at 4 °C. The cytosolic fractions were filtered through a 0.2 μ m syringe filter (Sartorius, Goettingen, Germany). 2 ml aliquots of cytosolic fractions were

kept as reference for experiments to monitor the purification at all steps. For investigation of the influence of heme supplement and additionally of lipid medium supplement 4 mg/l hemin or/and 1 % lipid medium supplement were added.

One-Step-Purification of NOsGC

The purification was performed on ice or at 4°C. The chromatographic step was performed on an ÄKTApurifier 100 system (GE Healthcare, Munich, Germany). Cytosolic fractions were immediately applied to a *Strep-Tactin[®] Superflow[®]* high capacity resin (2 ml Volume in a TricornTM 10/20 column) at 1 ml/min [31]. Buffer W contained 100 mM Tris-HCl, 1 M NaCl, 1 mM EDTA, 1 mM benzamidine, 10 mM DTT, pH 8.0. Buffer E was prepared by adding 2.5 mM D-desthiobiotin to buffer W. The column was washed ad 1 ml/min with buffer W for 5 column volumes (CV). With 5 CV buffer E the elution of the NOsGC was performed. Monitoring the absorbance at 254 nm, 280 nm and 430 nm showed a single peak in the fraction which contained NOsGC. Regeneration of the column was performed by 15 CV buffer R (100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM HABA, pH 8.0) at 1 ml/min and 8 CV of buffer W at 1 ml/min. The pooled fractions (approximately 15 ml) were concentrated to a volume of about 500 μ l using an Amicon Ultra-15 centrifugal filter unit with 30 kDa cut-off (Millipore, Schwalbach, Germany).

Determination of Protein Concentration and Guanylyl Cyclase Activity Assay

Protein concentrations were determined by the *Warburg-Christian* method [32] using a NanoPhotometerTM (Implen, Munich, Germany). Guanylyl cyclase activity was measured as described previously [3]. Purified NOsGC was diluted with 50 mM TEA-HCl, 10 mM DTT, 1 mM EDTA, 0.5 μ g/ μ l bovine serum albumin (Roth, Karlsruhe, Germany), pH 7.4, quick-frozen in liquid nitrogen with 10 % (v/v) glycerol and stored at -80°C. Enzyme activity of purified protein (50 ng of protein per assay tube) were determined by incubation for 10 min at 37°C in the presence of 1 mM cGMP, 0.5 mM [α -³²P]GTP (about 0.2 μ Ci), 3 mM MgCl₂, 50 mM TEA-HCl, pH 7.4, 0.25 g/liter creatine kinase, 5 mM creatine phosphate, and 1 mM 3-isobutyl-1-methylxanthine in a total volume of 100 μ l as described by *Schultz and Boehme* [33]. Reactions were started by the addition of protein and incubation at 37°C. All experiments were stopped by ZnCO₃ precipitation, and purification of the enzyme-formed cGMP was performed as described previously. Basal enzyme activity measurements were performed in the absence of NO or cinaciguat. Measurements of stimulated enzyme were performed in the presence of the NO donor DEA/NO or cinaciguat. DEA/NO was dissolved in 10 mM NaOH, which did not affect the enzyme activity (data not shown). Cinaciguat was dissolved in 100 % DMSO and then diluted in distilled water to a final concentration of 10 μ M so that the final DMSO concentration in the enzyme assay did not exceed 2.5% (v/v). At this concentration no effects of DMSO on enzyme activity were observed (data not shown).

SDS-polyacrylamide electrophoresis gels and immunoblot analysis

Aliquots of 50 μ g (cytosolic fractions) or 5 μ g (purified enzyme) protein were heated for 3 min at 105°C with lidheat of 120°C (PCR-Cycler) in a modified Laemmli sample buffer (50 mM Tris-HCl, 1% SDS, 100 mM DTT, 30% Glycerol, pH 7.5). After the heat-incubation 1 μ l of a blue sample puffer [10% (m/w) bromophenol blue solved in the modified Laemmli buffer] were

added and the probes were resolved on 10% slab gels. Proteins were stained according to Kang et al. 2002 [8]. As protein markers predominantly PageRuler™ Prestained Protein Ladder and PageRuler™ Unstained Protein Ladder from Fermentas (St. Leon-Rot, Germany) were used. For immunoblotting, protein fractions were transferred electrophoretically to a nitrocellulose membrane [Amersham™ Hybond™ ECL (GE Healthcare)]. The membrane was reversibly stained with Ponceau S to evaluate the protein transfer. Unspecific binding sites were saturated by immersing the membrane for 1 h at room temperature in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% (v/v) Tween 20) containing 5% nonfat dry milk. The following antibodies against the two NOsGC subunits were used for detection: anti- α_1 (1:5,000) (Sigma, G4280) or α_1 -1200 (1:1,000; as described in [3]) and anti- β_1 (1:4,000) (Sigma, G4530). Antibodies were incubated for 1 h in TBST-buffer at room temperature. The membranes were washed three times for 10 min with TBST and subsequently incubated for 1 h with horseradish-peroxidase-conjugated anti-rabbit IgG antibody (1:2,000/Cell Signaling Technology, distributor: New England Biolabs, Frankfurt am Main, Germany or 1:4,000/Sigma). After three washes with TBST-buffer the membranes were processed with the enhanced chemiluminescence western blotting detection system according to the recommendations of the manufacturer (Roche) and the signals were detected with a charge-coupled device camera (Intas, Goettingen, Germany) or the membranes were processed with ECL Western blotting detection system according to manufacturer's recommendations (Amersham Pharmacia Biotech, Piscataway, USA).

Quantification of heterodimerization

For quantification of subunit interaction, the Coomassie stained gels were either captured using a white trans-illuminator and a charge-coupled device camera (HighRes, Intas) or scanned using ScanMaker i900 (Microtek, Willich, Germany). The analysis was done using LabImage 1D (Kapelan, Leipzig, Germany). For the wild-type of the human α_1 -subunit and C- α_1 the densitometric values of α_1 full-length and the truncation were normalized to the value of β_1 in the same lane and dimerization is given as a percentage of α_1 WT dimerization, which was set to 100%. Western blots were analyzed accordingly with similar results (data not shown).

Fluorescence Lifetime Imaging (FLIM) using a confocal laser scanning microscope

Determination of fluorescence lifetime was done as described previously [9]. For microscopy HEK-293 cells were seeded in 24-well imaging plates with special glass bottom (zell-kontakt, Noerten-Hardenberg, Germany; distributed by PAA Laboratories, Coelbe, Germany) and transfected with the cDNA coding for the respective constructs for the expression of fluorescent tagged NOsGC subunits using Lipofectamin™ LTX (Invitrogen). 48 h post transfection cells were imaged at 37°C on a Nikon Ti-E microscope equipped with an incubation chamber (Okolab, Naples, Italy) using a 60-fold immersion objective (NA 1.4, Nikon). For live cell imaging culture medium was removed, cells were washed twice and supplemented with Hank's balanced salt solution.

Fluorescence decays were measured in cells expressing the FRET donor alone (ECFP) using the vector pECFP-N1 (Clontech) as a negative control. The rat α_1 -subunits, wild-type and amino-

terminal truncation are carboxy-terminally tagged with ECFP as FRET donor and the rat β_1 -subunit is carboxy-terminally tagged with FRET acceptor EYFP using the vector pEYFP-N1 (Clontech) or vice versa. Images were collected using a 405 nm pulsed laser. Emitted fluorescence signals were selected using a CFP bandpass filter (475/20 nm). FLIM images in the time domain from fluorescent cells were recorded with a 4 channel time gated detection system (LiMo module, Nikon). The images were 256×256 pixels in size and the acquisition time was 5 min.

Localization of α_1 constructs by confocal laser scanning microscope

The cells were prepared as described above. Amino-terminally ECFP-tagged heme oxygenase 1 (human, HO-1) was used as a marker for the endoplasmic reticulum [11].

Analyzing the redox state of the C- α_1 and the α_1 wild type protein

Using fusion proteins of the respective α_1 isoforms with the redox sensor Grx1-roGFP2 we performed a ratiometric measurement. The cells were simultaneously excited with a 405 nm and a 488 nm laser. The emission was collected by a GFP bandpass filter (525/50 nm). The ratio of the 405/488 emission corresponds to the redox state. A high value means a more oxidized state and vice versa.

Statistical analysis

The results are expressed as means \pm SEM of at least three independent experiments. All results were controlled for their statistical significance by Student's *t*-test. A value of $p < 0.01$ was considered to be statistically significant.

Supporting Information

Data S1 Amino acid sequence alignment of human (Accession number: NP_000847.2) and rat (Accession number: NP_058786.2) α_1 -subunit to show sequence differences (performed with ClustalW2 at www.ebi.ac.uk). Gaps are marked. (PDF)

Data S2 Nucleic acid sequence alignment (partly shown) of human α_1 full-length (GB: Y15723) and both splice-variants C*- α_1 (GB: BX649180)/C- α_1 (GB: AK226125) (performed with ClustalW2 at www.ebi.ac.uk). Initiation codons are marked. (PDF)

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Author Contributions

Conceived and designed the experiments: JRK SB. Performed the experiments: JRK MB TH ML. Analyzed the data: JRK SB. Contributed reagents/materials/analysis tools: NH MK. Wrote the paper: JRK SB.

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