

# Reactive Oxygen Species Induce Tyrosine Phosphorylation of and Src Kinase Recruitment to NO-sensitive Guanylyl Cyclase\*<sup>§</sup>

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Soluble guanylyl cyclase (sGC) is the major cytosolic receptor for nitric oxide (NO) that converts GTP into the second messenger cGMP in a NO-dependent manner. Other factors controlling this key enzyme are intracellular proteins such as Hsp90 and PSD95, which bind to sGC and modulate its activity, stability, and localization. To date little is known about the effects of posttranslational modifications of sGC, although circumstantial evidence suggests that reversible phosphorylation may contribute to sGC regulation. Here we demonstrate that inhibitors of protein-tyrosine phosphatases such as pervanadate and bisperoxo(1,10-phenanthroline)oxovanadate(V) as well as reactive oxygen species such as H<sub>2</sub>O<sub>2</sub> induce specific tyrosine phosphorylation of the  $\beta_1$  but not of the  $\alpha_1$  subunit of sGC. Tyrosine phosphorylation of sGC $\beta_1$  is also inducible by pervanadate and H<sub>2</sub>O<sub>2</sub> in intact PC12 cells, rat aortic smooth muscle cells, and in rat aortic tissues, indicating that tyrosine phosphorylation of sGC may also occur *in vivo*. We have mapped the major tyrosine phosphorylation site to position 192 of  $\beta_1$ , where it forms part of a highly acidic phospho-acceptor site for Src-like kinases. In the phosphorylated state Tyr(P)-192 exposes a docking site for SH2 domains and efficiently recruits Src and Fyn to sGC $\beta_1$ , thereby promoting multiple phosphorylation of the enzyme. Our results demonstrate that sGC is subject to tyrosine phosphorylation and interaction with Src-like kinases, revealing an unexpected cross-talk between the NO/cGMP and tyrosine kinase signaling pathways at the level of sGC.

NO-sensitive guanylyl cyclase or soluble guanylyl cyclase (sGC<sup>3</sup>; EC 4.6.1.2) is the principal cytosolic receptor for nitric oxide (NO) and converts GTP into the second messenger cGMP. Mammalian sGCs are obligate heterodimers of  $\alpha$  and  $\beta$  subunits, each comprising an N-terminal regulatory domain, a central region involved in dimerization, and a C-terminal catalytic domain (1–4). The  $\beta$  subunit accommodates a heme moiety holding Fe<sup>2+</sup> in a pentacoordinated state, with His-105 being the axial ligand (5, 6). Displacement of Fe<sup>2+</sup> from the protopor-

phyrin plane of the nitrosylated heme complex releases the constraint imposed by the His-105 coordination (7) and triggers a conformational change in the regulatory domain that propagates to the active site thereby enhancing catalytic efficiency of sGC (8). Activation of sGC results in the rapid increase of intracellular cGMP levels, and through the stimulation of its multiple downstream effectors cGMP mediates pleiotropic effects such as smooth muscle relaxation, inhibition of platelet aggregation and leukocyte adhesion, and modulation of cell proliferation and migration (9–11).

Given the key regulator role of sGC in cGMP-dependent pathways, one may anticipate that mechanisms beyond NO stimulation may exist that contribute to the fine-tuning of cGMP generation. For instance, regulated gene expression of the sGC subunits has been documented (12, 13), and an equilibrium between homo- and heterodimers has been claimed to balance sGC activity (14). Reversible protein-protein interactions of sGC with adaptor proteins such as PSD95 and chaperones like Hsp90, Hsp70, or the  $\eta$ -subunit of the chaperonin-containing t-complex can alter the activity, stability, and localization of sGC (15–18). Also, phosphorylation appears to be important for sGC activity regulation. For example, *in vitro* phosphorylation of Ser/Thr residues of the  $\alpha_1$  subunit of sGC by a cAMP-dependent protein kinase increased the activity of sGC (19), most likely through stabilization of the nitrosyl-heme complex (20). In PC12 cells Ser/Thr phosphorylation by Ca<sup>2+</sup>-dependent protein kinase enhanced sGC activity (21, 22), whereas cGMP-dependent protein kinase attenuated the catalytic capacity of sGC, most likely through an inhibitory feedback mechanism (23) via stimulation of protein phosphatase(s) which reduces the Ser/Thr phosphorylation level of  $\beta_1$  (24). Circumstantial evidence suggests that tyrosine phosphorylation may also contribute to sGC activity regulation in PC12 cells through 17 $\beta$ -estradiol-mediated activation of protein tyrosine phosphatases (PTPs) such as SHP-1 (25); however, Tyr phosphorylation of sGC has not been demonstrated to date.

Multiple signaling pathways are governed by the delicately balanced activities of protein-tyrosine kinases and PTPs, which govern cellular signaling through reversible Tyr phosphorylation (26). In the case of receptor-tyrosine kinases binding of the cognate ligands promotes the recruitment and phosphorylation of cytosolic substrates, often endowed with Src homology type 2 (SH2) and/or phosphotyrosine (Tyr(P)) binding domains. Alternatively ligand-independent stimulation of receptor-tyrosine kinases, *e.g.* by reactive oxygen species such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) or by UV light, may also trigger Tyr phosphorylation pathways (27, 28). Among the major downstream targets of H<sub>2</sub>O<sub>2</sub>-driven receptor-tyrosine kinase pathways are members of the Src kinase family, which can also be activated by H<sub>2</sub>O<sub>2</sub> (29–31). Activated Src-like kinases can phosphorylate multiple signal relay molecules and alter their structure or function by changing the catalytic activity, localization, and/or composition of signaling complexes through the recruitment of accessory proteins (32).

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<sup>3</sup> The abbreviations used are: sGC, soluble guanylyl cyclase; bpV(phen), bisperoxo(1,10-phenanthroline)oxovanadate(V); DETA, diethylenetriamine; EGF, epidermal growth factor; IBMX, isobutylmethylxanthine; NGF, nerve growth factor; PTP, protein-tyrosine phosphatase; PV, pervanadate; ROS, reactive oxygen species; SH2, Src homology domain 2; WT, wild type; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; GST, glutathione S-transferase; PBS, phosphate-buffered saline.

## Tyrosine Phosphorylation of Guanylyl Cyclase

Here we have set out to study the phosphorylation of human NO-sensitive guanylyl cyclase. We demonstrate Tyr phosphorylation of sGC in native and transfected cells as well as in intact aortic tissue. We show that both inhibition of PTPs and reactive oxygen species such as  $\text{H}_2\text{O}_2$  induce Tyr phosphorylation of sGC, thereby promoting the recruitment of Src-like kinases to the Tyr-phosphorylated  $\beta_1$  subunit. Our findings point to an unexpected cross-talk between NO/cGMP and tyrosine kinase signaling pathways at the level of sGC.

### EXPERIMENTAL PROCEDURES

**Materials**—DMEM, FCS, and penicillin/streptomycin were obtained from PAA (Pasching, Austria); gentamicin was from Invitrogen; elastase and collagenase were from Worthington (Lakewood, NJ); ECL<sup>TM</sup> detection reagents and glutathione-Sepharose<sup>TM</sup> 4B were from Amersham Biosciences; monoclonal antibody to c-Src (clone H-12) and protein A/G PLUS-agarose was from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal antibody to phosphotyrosine (P-Tyr-100, anti-pY) was from Cell Signaling (Beverly, MA); kinase inhibitor PP1 was from Biomol (Hamburg, Germany); kinase inhibitor PP2 and phosphatase inhibitor bisperoxo(1,10-phenanthroline)oxovanadate(V) (bpV(phen)) were from Calbiochem; complete protease inhibitor mixture was from Roche Applied Science. All other reagents including monoclonal antibody to vesicular stomatitis virus (clone P5D4), monoclonal antibody to GST, nerve growth factor (NGF), epidermal growth factor (EGF), phenylmethylsulfonyl fluoride, diethylenetriamine/nitric oxide (DETA/NO),  $\text{Na}_3\text{VO}_4$ , isobutylmethylxanthine (IBMX), ATP, xanthine, and xanthine oxidase were from Sigma.

**Antibody Production**—Antisera to the  $\alpha_1$  (AS587) and  $\beta_1$  subunits (AS566) were raised in rabbits using synthetic peptides unique for human sGC $\alpha_1$  (positions 94–121) and  $\beta_1$  (593–614), respectively. Antisera to the regulatory domain of human sGC $\alpha_1$  (1–419) and to the catalytic domain of  $\beta_1$  (404–619) were produced in rabbits (AS558 and AS556, respectively) or mice (AS613, AS614) with the corresponding GST fusion proteins as described (4, 33). Phospho-specific antiserum to  $\beta_1$ -pY-192 (AS680) was raised in rabbit using non-conjugated peptide  $\text{NH}_2$ -KEEDF(pY)EDLDRFEENGQTQESR-COOH (pY is phosphorylated tyrosine) as the antigen.

**Construction of Expression Plasmids**—The coding region of sGC $\alpha_1$  was amplified by PCR and subcloned into the EcoRI-XhoI sites of pSG8, *i.e.* a modified version of eukaryotic expression vector pSG5. The cDNA fragment of  $\beta_1$  was ligated into the EcoRI site of pEDmtxr. For the expression of vesicular stomatitis virus-tagged  $\beta_1$  or single domains thereof, we amplified the corresponding cDNA segments by PCR and subcloned them into pSG8 previously modified for in-frame expression of an N-terminal vesicular stomatitis virus tag. Single point mutations were introduced using the QuikChange XL site-directed mutagenesis Kit from Stratagene (La Jolla, CA). The cDNAs for wild type (WT) and dominant-negative mutant of chicken Src, pSG5-Src and pSG5-SrcK<sup>-</sup>, respectively, were generous gifts of Dr. Rudi Busse (University of Frankfurt, Frankfurt, Germany). All constructs were subjected to DNA sequencing before use.

**Transfection and Cell Culture**—COS-1 and PC12 cells were cultured in DMEM supplemented with 10% FCS. Transient transfections of COS-1 cells were done with DEAE-dextran. In brief, a 10-cm dish containing  $6 \times 10^5$  cells was washed with PBS, and expression plasmids were applied in 5.7-ml serum-free medium mixed with 300  $\mu\text{l}$  of DEAE-dextran (1 mg/ml) and 12  $\mu\text{l}$  chloroquine (100 mM). After incubation for 2.5 h, cells were treated with 10% Me<sub>2</sub>SO in PBS for 2 min and cultured in DMEM, 10% FCS for 24–48 h before use. To induce Tyr phosphorylation, cells were incubated for 20–30 min at 37 °C with 20  $\mu\text{M}$  bpV(phen) or 30 min with 100  $\mu\text{M}$  pervanadate (PV) in DMEM, 10% FCS

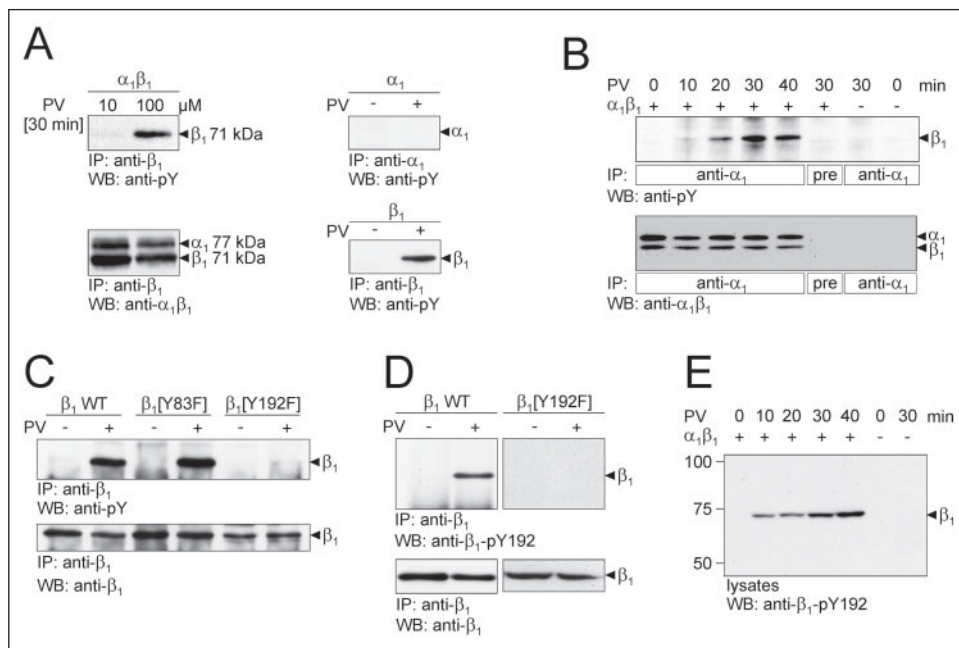
unless stated otherwise. The PV solution was freshly prepared by incubating 10 mM  $\text{Na}_3\text{VO}_4$  and 300 mM  $\text{H}_2\text{O}_2$  for 10 min at RT. To inhibit Src family tyrosine kinases, cells were incubated with PP1 or PP2 (final concentration 2.5–10  $\mu\text{M}$ , as indicated) for 1 h followed by incubation with 20  $\mu\text{M}$  bpV(phen) for 30 min. To induce oxidative stress,  $\text{H}_2\text{O}_2$  was added to the medium at the indicated concentrations for 2–20 min. Alternatively, cells were incubated with 2.5 milliunits/ml xanthine oxidase in the absence (control) or presence of 100  $\mu\text{M}$  xanthine. Before stimulation with 10 ng/ml EGF or 50 ng/ml NGF, cells were starved overnight in DMEM, 0.5% FCS. SYF, *i.e.* cells deficient in Src, Yes, and Fyn, and Src<sup>++</sup>, *i.e.* cells deficient in Yes and Fyn but retaining normal Src levels (ATCC, Manassas, VA) were cultured in DMEM supplemented with 10% FCS. For transient transfections, 5-cm dishes containing  $8 \times 10^5$  Src, Yes, and Fyn or  $4 \times 10^5$  Src<sup>2+</sup>, cells were treated with Nanofectin (PAA) and expression plasmids coding for sGC $\alpha_1$  and sGC $\beta_1$  and incubated for 24 h before use. Tyr phosphorylation was induced as detailed above.

**Immunoprecipitation and Western Blotting**—Immunoprecipitation was done as described (33). Briefly, cells were lysed with immunoprecipitation buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 50 mM NaF, 25 mM  $\text{Na}_4\text{P}_2\text{O}_7$ , 1 mM  $\text{Na}_3\text{VO}_4$ , 40 mM  $\beta$ -glycerophosphate, 1 mM phenylmethylsulfonyl fluoride supplemented with protease inhibitor mixture), and the cleared lysate was incubated with antiserum. After immunoprecipitation samples were subjected to SDS-PAGE and analyzed by Western blotting (33).

**GST Fusion Proteins and GST Pull-down Assay**—Bacterial expression plasmids encoding the SH3 domain alone or GST fusions of the SH2/SH3 domains combined were a kind gift of Dr. Ivan Dikic (University of Frankfurt, Germany). The cDNAs encoding the unique SH2 domains of tyrosine kinases Src, Fyn, and Abl or the second SH2 domain of phospholipase C $\gamma$ 1 were amplified by PCR and cloned into pGEX2T. The single point mutation (R175G) was introduced into the Src SH2 domain using the QuikChange XL site-directed mutagenesis kit from Stratagene. All constructs were verified by DNA sequencing. Bacterial GST fusion proteins were expressed in *Escherichia coli* BL21 for 4 h at 30 °C after induction with 100  $\mu\text{M}$  isopropyl 1-thio- $\beta$ -D-galactopyranoside. Cells were pelleted, washed with PBS, resuspended in PBS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and lysed by sonification. After centrifugation at 20,000  $\times g$  for 15 min, glutathione-Sepharose beads were added to the cleared lysate, and the mixture was incubated for 1 h at 4 °C on a rotating wheel. Beads were washed 4 $\times$  with PBS, and Sepharose-bound GST fusion proteins were incubated for 3 h at 4 °C with Triton X-100-soluble extracts from COS-1 cells overexpressing WT  $\beta_1$ , mutant  $\beta_1$ [Y192F], or WT  $\alpha_1\beta_1$  pretreated with or without 100  $\mu\text{M}$  PV for 30 min. Beads were washed 4 $\times$  with immunoprecipitation buffer, and bound proteins were eluted and analyzed by Western blotting (33).

**In Vitro Phosphorylation**—For in vitro phosphorylation, the cDNA comprising position 133–250 of  $\beta_1$  was amplified by PCR and cloned into pGEX2T vector. Glutathione-Sepharose beads coupled to 10  $\mu\text{g}$  of purified GST-GC $\beta_1$ -(133–250) (see above) were washed with labeling buffer (50 mM Hepes, pH 7.5, 100  $\mu\text{M}$  EDTA, 0.05% (v/v) Triton X-100, 100  $\mu\text{g}/\text{ml}$  BSA) and resuspended in 45  $\mu\text{l}$  of kinase buffer (30 mM Hepes, pH 7.5, 60  $\mu\text{M}$  EDTA, 0.03% (v/v) Triton X-100, 60  $\mu\text{g}/\text{ml}$  BSA, 100  $\mu\text{M}$  ATP, 10 mM  $\text{MgCl}_2$ ). The reaction was started by adding 1.5 units of recombinant Src (Upstate Biotechnology, Lake Placid, NY) to the suspension. After incubation for 2.5 h at 37 °C, the beads were washed 3 $\times$  with labeling buffer. Protein was eluted and subjected to SDS-PAGE followed by Western blotting with anti-PY.

**FIGURE 1. Pervanadate induces Tyr phosphorylation of sGC $\beta_1$ .** COS-1 cells were transfected with cDNAs encoding WT human sGC $\alpha_1$  and  $\beta_1$  singly (A, C, and D) or combined (A, B, and E) or mutants  $\beta_1$ [Y83F] and  $\beta_1$ [Y192F] as indicated (C and D). Cells were incubated in the absence (–) or presence (+) of 100  $\mu$ M PV for 30 min unless stated otherwise. Representatives of at least three independent experiments are shown throughout. Immunoprecipitation (IP) was done with antibodies to  $\alpha_1$  (AS587) or  $\beta_1$  (AS566) followed by Western blotting (WB to P-Tyr-100) (A, B, and C), sGC (anti- $\alpha_1\beta_1$ ; mixture of AS613 and AS614; A and B),  $\beta_1$  (AS566 in C, AS614 in D), or phospho-specific antiserum  $\beta_1$ -pY-192 (AS680; D and E). For a control the corresponding pre-immune (pre) serum was used. Alternatively, total cell lysates from COS-1 cells overexpressing  $\alpha_1\beta_1$  were analyzed by Western blotting with anti- $\beta_1$ -pY-192 (AS680). Proteins are identified on the right.



**Tissue Preparation and Isolation of Rat Aortic Vascular Smooth Muscle Cells**—Anesthetized 6-week-old Wistar rats were sacrificed, and aortae were excised, cleaned from connective tissue, and placed in PBS. Aortae were cut in rings of ~3-mm height and placed in DMEM, 10% FCS with or without 100  $\mu$ M PV for 1 h. Medium was aspirated, tissues were frozen in liquid nitrogen and minced mechanically, and immunoprecipitation of sGC subunits was done as detailed above. Alternatively, aortae were cut into small pieces and placed in 500  $\mu$ l of digestion medium (DMEM including 20 mM HEPES, 2 mg/ml collagenase, 0.18 mg/ml elastase, 1 mg/ml BSA, 50  $\mu$ g/ml gentamicin) for 30 min. After centrifugation at 800  $\times$  g for 3 min, the pellet was resuspended in 500  $\mu$ l of digestion medium and further incubated for 2 h. The reaction was stopped by adding 5 ml of DMEM, 10% FCS. Cells were centrifuged as above, resuspended in 2 ml of DMEM, 10% FCS, 50  $\mu$ g/ml gentamicin, and seeded on a 3-cm dish. Three days after isolation medium was changed to DMEM, 10% FCS containing penicillin/streptomycin.

**sGC Activity Assay**—COS-1 cells were transfected with  $\alpha_1$  and  $\beta_1$  or  $\beta_1$ [Y192F], and 24 h later the cells were incubated for 20 min at 37  $^{\circ}$ C with or without 20  $\mu$ M bpV(phen) in HEPES-buffered DMEM, 10% FCS. To monitor sGC activity we added phosphodiesterase inhibitor isobutylmethylxanthine IBMX (final concentration 1 mM) 10 min before stimulation. Cells were stimulated with DETA/NO, diluted in assay buffer (15 mM Tris/HCl, pH 7.4, 1.2 mM  $\text{Na}_2\text{HPO}_4$ , 1.2 mM  $\text{MgSO}_4$ , 1.5 mM  $\text{CaCl}_2$ , 3 mM KCl, 130 mM NaCl, 11 mM glucose, 1 mM IBMX) in the presence or absence of 20  $\mu$ M bpV(phen) for 3 min and lysed with 0.1 M HCl. The acid extracts were collected, and intracellular cGMP was quantified using the cyclic GMP (low pH) immunoassay (R&D Systems, Wiesbaden, Germany).

**Data Analyses and Statistics**—Data are expressed as the means  $\pm$  S.E. Statistical significance was tested by Student's *t* test and set at  $p < 0.01$  (\*\*\*). Potential phosphorylation sites were identified by the NetPhos 2.0 program (Center for Biological Sequence Analysis, Technical University of Denmark).

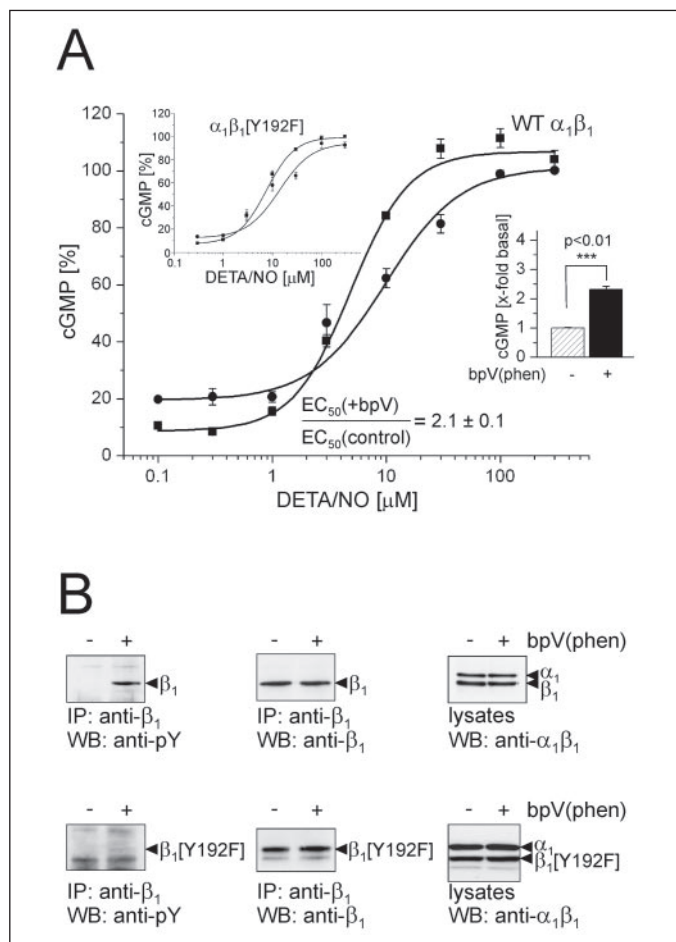
## RESULTS

**Pervanadate Treatment Reveals Tyr Phosphorylation of the  $\beta_1$  Subunit of Human sGC**—To screen for Tyr phosphorylation of sGC, we overexpressed the  $\alpha_1$  and  $\beta_1$  subunits of human sGC in COS-1 cells, singly or combined. The cells were incubated in the absence or presence

of the broad-specificity PTP inhibitor PV at 100  $\mu$ M for 5–40 min or at the indicated concentrations for 30 min. After cell lysis immunoprecipitation was done with subunit-specific antibodies followed by Western blotting with anti-pY. A dose- and time-dependent Tyr phosphorylation peaking around 30 min was observed for the  $\beta_1$  but not for the  $\alpha_1$  subunit of sGC (Fig. 1, A and B; supplemental Fig. S1). Under the same conditions we observed phosphorylation of the singly expressed  $\beta_1$  subunit but not of  $\alpha_1$  (Fig. 1A, right panel) and demonstrated that phosphorylation of  $\beta_1$  also occurs in the holoenzyme, *i.e.* when  $\beta_1$  is associated with  $\alpha_1$  (Fig. 1B). To map the phosphorylated region(s) in  $\beta_1$  we used vesicular stomatitis virus-tagged constructs harboring the regulatory or catalytic domains of  $\beta_1$ , *i.e.*  $\beta_1$ -(1–348) and  $\beta_1$ -(349–619), respectively. Incubation of transfected cells with PV specifically revealed Tyr phosphorylation of the regulatory but not the catalytic domain of  $\beta_1$  (supplemental Fig. S2). Thus, inhibition of PTPs by PV reveals Tyr phosphorylation of the regulatory domain of sGC $\beta_1$ .

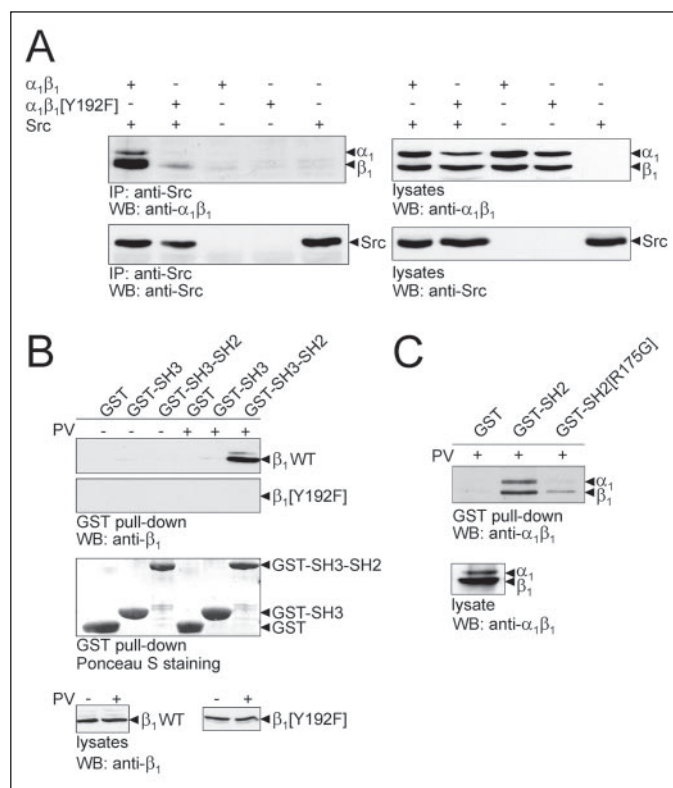
**PTP Inhibition Modulates NO-induced sGC Activity**—We wondered whether Tyr phosphorylation of  $\beta_1$  might have an impact on sGC activity. To exclude a direct effect on sGC activity by  $\text{H}_2\text{O}_2$  (which is required for the *in situ* production of PV), we employed the PTP inhibitor bpV(phen), which also induced sGC $\beta_1$  phosphorylation without affecting the level of total sGC protein (Fig. 2B, upper panels). We stimulated COS-1 cells expressing human  $\alpha_1\beta_1$  with increasing concentrations of the NO donor DETA/NO in the presence of phosphodiesterase inhibitor IBMX (1 mM) and monitored the generation of cGMP (Fig. 2A). Incubation of  $10^5$  cells with 0.3 and 100  $\mu$ M DETA/NO for 3 min resulted in the production of  $4.5 \pm 0.3$  and  $60 \pm 1.8$  pmol of cGMP, respectively. The concentration-response curves recorded with or without bpV(phen) revealed two prominent differences; the presence of PTP inhibitor increased basal sGC activity by a factor of  $2.3 \pm 0.1$  (Fig. 2A, right inset) and shifted the concentration-response curve for DETA/NO to the right such that the corresponding  $\text{EC}_{50}$  value more than doubled ( $12.3 \pm 1.4$   $\mu$ M versus  $5.9 \pm 0.7$   $\mu$ M under control conditions). Thus, both cyclase activity and NO responsiveness of sGC are moderately affected by the presence of PTP inhibitors.

**The Major Phosphorylation Site of sGC $\beta_1$  Is at Tyr-192**—To test whether Tyr phosphorylation is causally linked to activity modulation of sGC, we mapped the phosphorylation site(s) present in the regulatory domain of  $\beta_1$ . Of 13 Tyr residues, Tyr-83 and Tyr-192 were predicted by



**FIGURE 2. NO-induced sGC activity is inhibited by Tyr phosphorylation in COS-1 cells.** *A*, COS-1 cells expressing WT  $\alpha_1\beta_1$  or mutant  $\alpha_1\beta_1$ [Y192F] (*left inset*) stimulated with increasing concentrations of DETA/NO for 3 min in the presence of 1 mM IBMX, with (●) or without (■) before incubation with 20  $\mu\text{M}$  bpV(phen) for 20 min. Media were aspirated, and 0.1 M HCl was added to extract cGMP. The extracts were collected, and cGMP concentrations were quantified (given as percent of cGMP level at 500  $\mu\text{M}$  DETA/NO). Changes of the basal activity of sGC in the presence of 20  $\mu\text{M}$  bpV(phen) are given as  $\times$ -fold over control in the absence of the inhibitor (*right inset*). *B*, COS-1 cells expressing  $\alpha_1\beta_1$  (*top panel*) or  $\alpha_1\beta_1$ [Y192F] (*bottom panel*) were incubated with (+) or without (-) 20  $\mu\text{M}$  bpV(phen). Cells were lysed and immunoprecipitated (IP) with anti- $\beta_1$  (AS566) followed by Western blotting (WB) with anti-pY, anti- $\beta_1$  (AS614), or antibodies to  $\alpha_1\beta_1$  (mixture of AS566 and AS587). Data are the means  $\pm$  S.E. from three consecutive measurements; representatives of three independent experiments are shown.

the NetPhos algorithm to be primary target sites for tyrosine kinases. Mutants of  $\beta_1$ , where Phe substituted for Tyr, were overexpressed in COS-1 cells in the absence or presence of PV. Under these conditions phosphorylation of  $\beta_1$ [Y83F] was similar to that of WT  $\beta_1$ , whereas phosphorylation of  $\beta_1$ [Y192F] was markedly reduced (Fig. 1C). Thus, it appears that PV can induce phosphorylation of Tyr residues in the regulatory subunit of sGC $\beta_1$  and that the major although not exclusive phosphorylation site is at residue Tyr-192. To substantiate this finding, we generated an antibody specific for Tyr(P)-192 and found that it specifically detects  $\beta_1$  phosphorylation in the presence but not in the absence of PV both in immunoprecipitates of the singly expressed  $\beta_1$  subunit (Fig. 1D) and in total cell lysates of the  $\alpha_1\beta_1$  holoenzyme (Fig. 1E and supplemental Fig. S3). To test whether Tyr phosphorylation of Tyr-192 is responsible for sGC activity modulation we co-expressed WT  $\alpha_1$  and mutant  $\beta_1$ [Y192F] in COS-1 cells. Stimulation of mutant  $\alpha_1\beta_1$ [Y192F] with 0.3  $\mu\text{M}$  and 100  $\mu\text{M}$  DETA/NO resulted in the production of  $4.7 \pm 0.1$  and  $58 \pm 1.2$  pmol/ $10^5$  cells of cGMP, respectively. Treatment with bpV(phen) induced only minor Tyr phosphorylation of the mutant enzyme (Fig. 2B, *bottom panels*), yet the concentration-

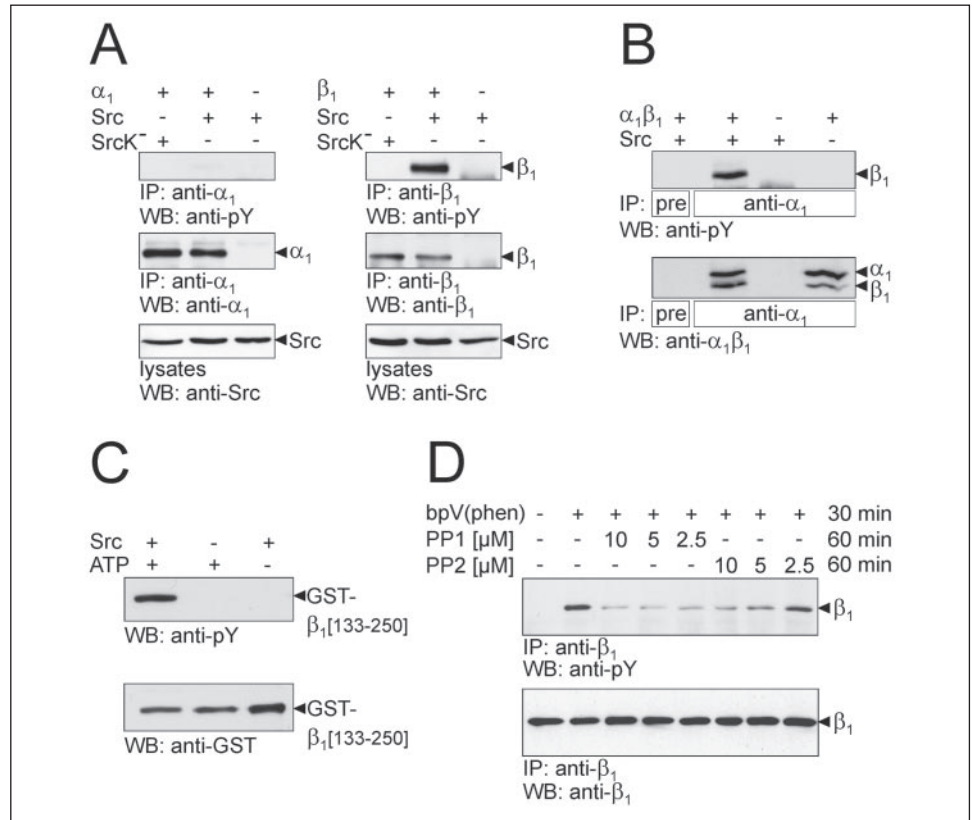


**FIGURE 3. Src interacts with sGC via its SH2 domain.** *A*, COS-1 cells overexpressing Src together with  $\alpha_1\beta_1$  or mutant  $\alpha_1\beta_1$ [Y192F] were lysed and immunoprecipitated (IP) with anti-Src followed by Western blotting (WB) with anti- $\alpha_1\beta_1$  (mixture of AS613 and AS614) or anti-Src. To monitor protein expression, Western blots of the corresponding lysates were made with antibodies to  $\alpha_1\beta_1$  or anti-Src. Triton X-100-soluble extracts of COS-1 cells expressing WT  $\beta_1$  or mutant  $\beta_1$ [Y192F] (*B*) or WT  $\alpha_1\beta_1$  (*C*) pretreated with (+) or without (-) PV for 30 min were incubated with glutathione 4B-Sepharose beads previously bound to GST, GST fused to the SH3 domain (GST-SH3), or to the SH3 and SH2 domains of Src (GST-SH3-SH2) (*B*) and GST or GST fused to the SH2 domain (GST-SH2) or to mutant SH2 domain (GST-SH2[R175G]) of Src (*C*). Bound proteins were eluted with SDS sample buffer and analyzed by Western blotting with anti- $\beta_1$  (*B*, AS566) or anti- $\alpha_1\beta_1$  (*C*, mixture of AS566 and AS587). Expression of  $\alpha_1\beta_1$  or  $\beta_1$  constructs was monitored in lysates using anti- $\alpha_1\beta_1$  or anti- $\beta_1$ . Total protein was visualized by Ponceau S staining (*B*). Representatives of at least three independent experiments are shown.

response curves for  $\alpha_1\beta_1$ [Y192F] in the absence or presence of bpV(phen) did not significantly differ from those for WT  $\alpha_1\beta_1$  (Fig. 2A, *left inset*). Thus, it appears that PTP inhibition affects sGC activity independently of Tyr-192 phosphorylation.

**Tyr-192 Phosphorylation Creates a SH2 Docking Site in  $\beta_1$** —We wondered about alternative consequence(s) of Tyr-192 phosphorylation. Detailed inspection of the  $\beta_1$  sequence revealed that Tyr-192 residue is embedded in a cluster of negatively charged amino acid residues, <sup>188</sup>EEDFYEDLD, and that the phosphorylated motif of pYEDL (pY is phosphorylated tyrosine) could serve as a potential docking site for SH2 domains of Src-like kinases (34). To test this hypothesis we used lysates of COS-1 cells co-expressing sGC and Src and found that  $\alpha_1\beta_1$  co-immunoprecipitates with Src (Fig. 3A and supplemental Fig. S4). To substantiate this finding we employed GST fusion proteins of SH2 and/or SH3 domains of Src to pull down WT  $\beta_1$  or mutant  $\beta_1$ [Y192F] from COS-1 cell lysates in the absence or presence of PV. Under these conditions the constructs encompassing SH2/SH3 combined or SH3 alone failed to bind to non-phosphorylated WT  $\beta_1$  or mutant  $\beta_1$ [Y192F], whereas in the presence of PV the SH2/SH3 but not the SH3 fusion protein pulled down WT  $\beta_1$  but not  $\beta_1$ [Y192F] (Fig. 3B). We were able to reproduce this interaction with the isolated SH2 domain of Src and the sGC holoenzyme. Mutant SH2[R175G], where a crucial arginine residue responsible for binding the phosphate group was mutated to glycine, showed only weak affinity for phospho- $\beta_1$  (Fig. 3C). Using

**FIGURE 4. Src-like kinases are involved in  $\beta_1$  phosphorylation.** COS-1 cells expressing  $\alpha_1$ ,  $\beta_1$  (A) or  $\alpha_1\beta_1$  (B) together with WT Src (A and B) or catalytically inactive mutant SrcK<sup>-</sup> (A) were lysed and immunoprecipitated (IP) with antibodies to  $\alpha_1$  (AS587) or  $\beta_1$  (AS566). For the control the corresponding preimmune serum (*pre*) was used (B). Western blotting (WB) was done with anti-pY (A and B), anti- $\alpha_1$  (A, AS587), anti- $\beta_1$  (A, AS566), or anti- $\alpha_1\beta_1$  (B, mixture of AS613 and AS614). C, GST-GC $\beta_1$ -(133–250) bound to glutathione-Sepharose beads was incubated with recombinant Src in the presence or absence of ATP. Protein was eluted and subjected to SDS-PAGE followed by Western blotting with anti-PY (*top*) and anti-GST (*bottom*). D, COS-1 cells transfected with WT  $\beta_1$  were preincubated for 1 h with vehicle or increasing concentrations (2.5–10  $\mu$ M) of Src inhibitors PP1 and PP2, as indicated, followed by 20  $\mu$ M bpV(phen) for 30 min. Cells were lysed,  $\beta_1$  was immunoprecipitated with anti- $\beta_1$  (AS566), and Western blots were done with anti-pY (*top*) or anti- $\beta_1$  (AS614, *bottom*).



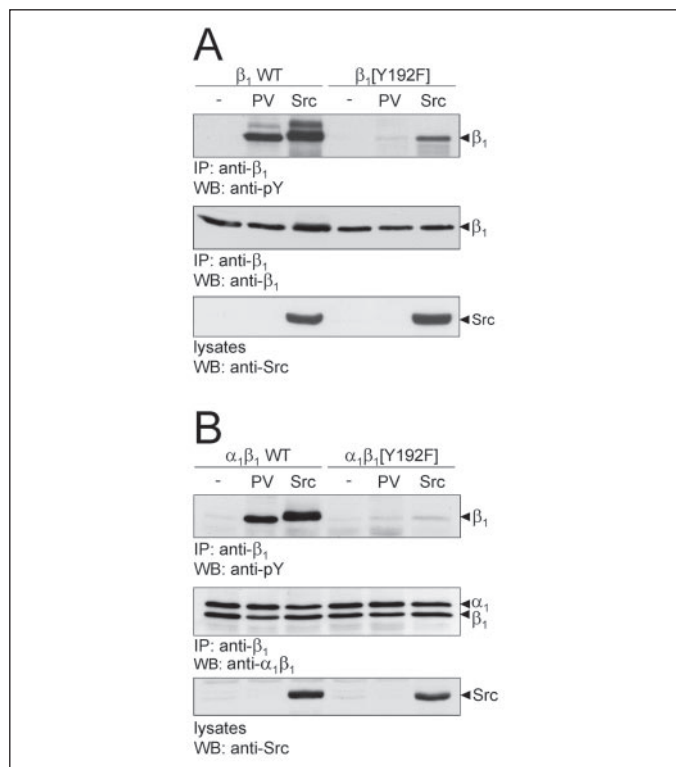
SH2 domains derived from Src-like kinase Fyn, unrelated tyrosine kinase Abl, or phospholipase C $\gamma$ 1 we found that Fyn-SH2 bound strongly to phosphorylated WT  $\beta_1$  but not to mutant  $\beta_1$ [Y192F], whereas the SH2 domains of Abl and phospholipase C $\gamma$ 1 failed to bind to WT or mutant  $\beta_1$  regardless their phosphorylation status (supplemental Fig. S5). Consistent with the results from pull-down assays, our co-immunoprecipitation experiments demonstrated strong binding of WT  $\alpha_1\beta_1$  holoenzyme to Src, whereas mutant  $\alpha_1\beta_1$ [Y192F] had little if any affinity for the kinase (Fig. 3A). Therefore, we conclude that Tyr(P)-192 provides a docking site for Src-like kinases at the regulatory domain of  $\beta_1$  subunit of sGC.

**Src-like Kinases Phosphorylate sGC $\beta_1$ .**—Next we asked which Tyr kinase(s) is involved in sGC $\beta_1$  phosphorylation. We noted that the <sup>188</sup>EEDFYEDLD motif of  $\beta_1$  matches the consensus sequence predicted for phospho-acceptor sites for Src kinase family members (35) and, therefore, tested whether Src might be involved in  $\beta_1$  phosphorylation. Co-expression of  $\alpha_1$  or  $\beta_1$  in COS-1 cells with Src or inactive kinase-dead mutant Src[K295M] (SrcK<sup>-</sup>) resulted in a strong phosphorylation of  $\beta_1$  but not of  $\alpha_1$  by WT Src but not by SrcK<sup>-</sup> (Fig. 4A), mirroring the target specificity observed for PV-induced phosphorylation. Not unexpectedly, overexpressed Src also phosphorylated the  $\alpha_1\beta_1$  holoenzyme in COS-1 cells (Fig. 4B) and recombinant Src phosphorylated GST- $\beta_1$ -(133–250) *in vitro* (Fig. 4C). To demonstrate the role of Src kinase(s) for sGC phosphorylation in a cellular context, we used COS-1 cells endogenously expressing Src-like kinases (36, 37), transfected them with WT  $\beta_1$ , and tested for bpV(phen)-induced Tyr phosphorylation of  $\beta_1$  in the presence of increasing concentrations of inhibitors PP1 and PP2 (Fig. 4D). Both PP1 and PP2 reduced  $\beta_1$  phosphorylation in a dose-dependent manner, albeit with different efficacies. To zoom in on potential kinases involved in sGC Tyr phosphorylation, we used cells generated from mouse embryos deficient for Src, Yes, and Fyn (SYF) or for Yes and Fyn only and expressing endogenous Src (Src<sup>+/+</sup>). We transiently transfected these with human  $\alpha_1\beta_1$  and incubated them in the presence or

absence of PV. After lysis and immunoprecipitation we clearly found phosphorylation of  $\beta_1$  in both cell types (supplemental Fig. S6), indicating that kinases other than Src, Yes, and Fyn must effect  $\beta_1$  phosphorylation in these cells. Combined, these results indicate that Src-like kinases are involved in  $\beta_1$  phosphorylation of sGC and that Tyr-192 in the regulatory domain of  $\beta_1$  appears to serve a dual role, *i.e.* in the unphosphorylated state it forms part of a phospho-acceptor site, whereas in the phosphorylated state it exposes a docking site for kinases such as Src and Fyn.

**sGC Is Phosphorylated on Multiple Sites.**—Because  $\beta_1$ [Y192F] shows minor although significant phosphorylation in the presence of PV (Fig. 1C), we asked whether this mutant can be phosphorylated by Src. Co-expression of WT  $\beta_1$  or  $\beta_1$ [Y192F] with Src in COS-1 cells resulted in a strong phosphorylation of WT  $\beta_1$  that was comparable with the PV-induced phosphorylation of  $\beta_1$  (Fig. 5A). Src also phosphorylated mutant  $\beta_1$ [Y192F] although to a lower extent than the WT  $\beta_1$ , pointing to the existence of phosphorylation site(s) other than Tyr-192. To check whether these potential sites are accessible in the holoenzyme, we repeated the experiment in the presence of  $\alpha_1$ . Interestingly enough,  $\beta_1$ [Y192F] phosphorylation was almost absent under these conditions (Fig. 5B), suggesting that heterodimerization may shield the kinase substrate site(s), which is only available in the  $\beta_1$  subunit when expressed alone. We also noted a Src- but not PV-induced shift of phosphorylated WT  $\beta_1$  toward higher molecular masses both for  $\beta_1$  alone and the  $\alpha_1\beta_1$  heterodimer, whereas no such shift was seen for the phosphorylated mutant  $\beta_1$ [Y192F] (Fig. 5, A and B, upper panels). These findings seem to support our notion that Src effects multiple phosphorylation of  $\beta_1$ .

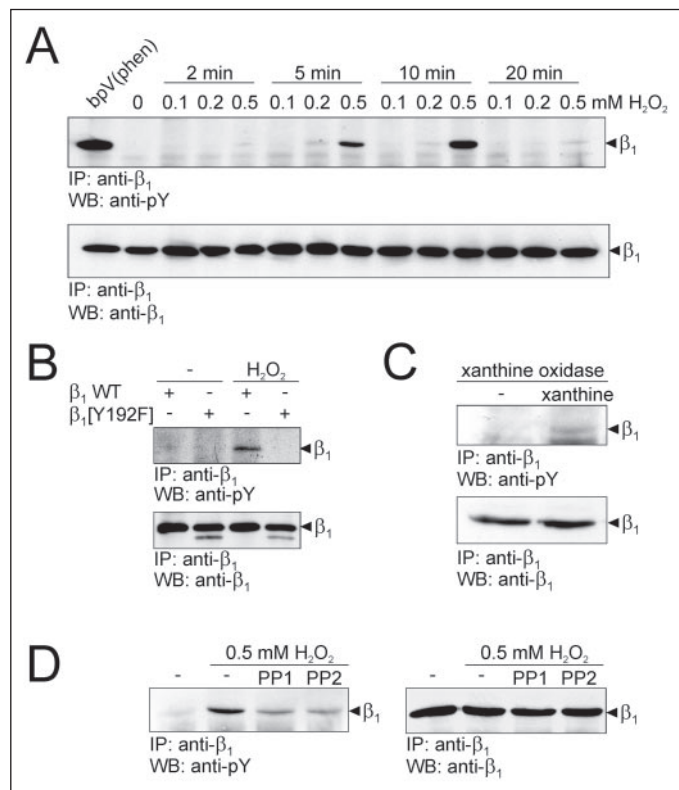
**Reactive Oxygen Species Induce  $\beta_1$  Phosphorylation.**—Next, we set out to identify physiological stimuli that may induce Tyr phosphorylation of sGC. In the cardiovascular system, reactive oxygen species (ROS) such as H<sub>2</sub>O<sub>2</sub> often trigger tyrosine kinase pathways, *e.g.* through the activation of Src, leading to phosphorylation of down-stream targets (31, 38). To test this possibility we exposed COS-1 cells overexpressing  $\beta_1$  to



**FIGURE 5. sGC is phosphorylated on multiple sites.** COS-1 cells expressing  $\beta_1$  or mutant  $\beta_1$ [Y192F] alone (A) or in combination with  $\alpha_1$  (B) were incubated in the absence (–) or presence of 100  $\mu$ M PV for 30 min; alternatively, cells were co-transfected with WT Src and incubated in the absence of PV. Cells were lysed, and  $\beta_1$  was immunoprecipitated (IP) with anti- $\beta_1$  (A5566) followed by Western blotting (WB) with anti-pY (top), anti- $\beta_1$  (A, AS614), or anti- $\alpha_1\beta_1$  (B, mixture of AS613 and AS614). Representatives of at least three independent experiments are shown.

increasing concentrations (0.1–0.5 mM) of  $H_2O_2$  and followed the phosphorylation pattern of  $\beta_1$  over time (Fig. 6A). A dose-dependent increase in  $\beta_1$  phosphorylation was obvious that appeared after 5 min, peaked at 10 min, and then quickly faded so that it was almost undetectable after 20 min of incubation (supplemental Fig. S7). Under the same conditions mutant  $\beta_1$ [Y192F] failed to show significant phosphorylation, indicating that Tyr-192 is the major target site for  $H_2O_2$ -driven phosphorylation of  $\beta_1$  (Fig. 6B). Notably, ROS formation and elimination are well balanced under physiological conditions, and disequilibrium may lead to severe oxidative stress (39). To mimic these conditions and to reveal the role of oxidative stress in sGC phosphorylation, we employed xanthine oxidase, producing both superoxide and hydrogen peroxide. Under these conditions  $\beta_1$  was indeed Tyr-phosphorylated (Fig. 6C). The time course of  $\beta_1$  phosphorylation was almost indistinguishable from that induced by direct application of  $H_2O_2$ , *i.e.* peaking at 10 min and fading after 20 min of incubation (supplemental Fig. S8). Thus, ROS such as  $H_2O_2$  are prime candidates for physiological effectors triggering sGC Tyr phosphorylation. To test whether Src-like kinases are involved in  $H_2O_2$ -mediated phosphorylation of sGC, we incubated COS-1 cells expressing  $\beta_1$  in the presence of kinase inhibitors PP1 and PP2 before stimulation with  $H_2O_2$ . The presence of PP1/2 significantly attenuated  $\beta_1$  phosphorylation (Fig. 6D), demonstrating that  $H_2O_2$ -induced phosphorylation likely involves activation of Src-like kinases.

**Phosphorylation of sGC Occurs in Intact Cells and Tissues**—To demonstrate sGC phosphorylation in native cells, we employed rat pheochromocytoma cells (PC12) which express significant amounts of endogenous sGC (Fig. 7A) and are sensitive to EGF and NGF (40). Stimulation of these cells with 50  $\mu$ M DETA/NO for 3 min in the pres-

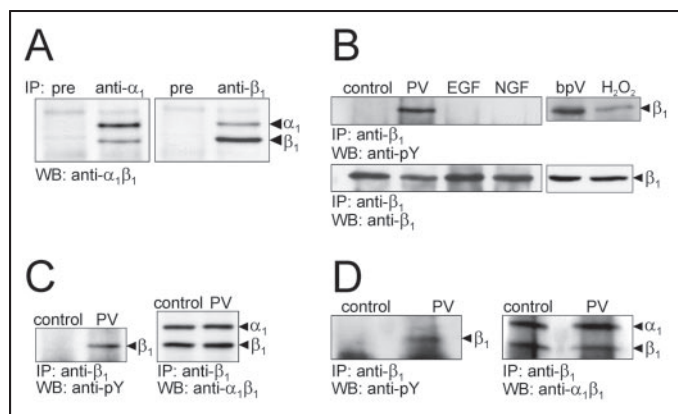


**FIGURE 6. Hydrogen peroxide induces phosphorylation of  $\beta_1$ .** A, COS-1 cells expressing  $\beta_1$  were incubated in the absence or presence of increasing concentrations (0.1–0.5 mM) of  $H_2O_2$  for varying time periods (2–20 min). For the control, cells were incubated for 30 min with 20  $\mu$ M bpV(phen) (A, outer left lane). B, COS-1 cells expressing  $\beta_1$  or  $\beta_1$ [Y192F] were incubated in the absence or presence of 0.5 mM  $H_2O_2$  for 10 min. COS-1 cells expressing  $\beta_1$  were incubated for 10 min with xanthine oxidase in the absence (–) or presence of xanthine (C) and for 1 h with vehicle or 10  $\mu$ M of PP1 and PP2 followed by incubation with 0.5 mM  $H_2O_2$  for 10 min (D). Cells were lysed,  $\beta_1$  was immunoprecipitated (IP) with anti- $\beta_1$  (A5566), and Western blotting (WB) was done with anti-pY or anti- $\beta_1$  (AS614) throughout.

ence of phosphodiesterase inhibitor IBMX resulted in a 36-fold increase of the intracellular cGMP concentration (basal,  $1 \pm 0.1$  pmol/ $10^6$  cells; stimulated,  $36 \pm 2$  pmol/ $10^6$  cells). Application of 20 mM  $H_2O_2$  for 10 min induced Tyr phosphorylation of  $\beta_1$ , albeit to a lower extent than PTP inhibitors bpV(phen) and PV, whereas application of 10 ng/ml EGF or 50 ng/ml NGF was without effect on  $\beta_1$  phosphorylation (Fig. 7B). Because UVC light has been shown to be a relevant source of oxidative stress leading to Tyr kinase activation (27), we irradiated PC12 cells with 1 J/m<sup>2</sup> UVC and detected incremental but significant phosphorylation of  $\beta_1$  but not of the  $\alpha_1$  subunit of sGC (data not shown). Finally, we tested whether  $\beta_1$  phosphorylation may also occur *ex vivo*, *e.g.* in animal tissues. To this end we incubated rings of rat aorta in the absence or presence of PV for 1 h and lysed the tissue. Immunoprecipitation and Western blotting revealed that  $\beta_1$  undergoes Tyr phosphorylation in the presence of PV (Fig. 7C). Along the same lines we probed for  $\beta_1$  Tyr phosphorylation in freshly isolated aortic smooth muscle cells from young rats and found a significant level of  $\beta_1$  phosphorylation upon PTP inhibition (Fig. 7D). Thus, it appears likely that Tyr phosphorylation of sGC may also occur *in vivo*.

## DISCUSSION

NO-sensitive guanylyl cyclases form a small family of heterodimeric heme proteins lacking transmembrane regions, which represent the principal intracellular NO receptors and regulate a wide array of cellular functions mostly through the modulation of cGMP-dependent kinases and phosphodiesterases and of cGMP-gated ion channels (9). Although



**FIGURE 7. Tyrosine phosphorylation of endogenous sGC occurs in cultured cells and organs.** *A*, PC12 cells were lysed, and endogenous  $\alpha_1\beta_1$  was immunoprecipitated (IP) using antibodies to  $\alpha_1$  (AS558) or  $\beta_1$  (AS556) followed by Western blotting (WB) with anti- $\alpha_1\beta_1$  (mixture of AS613 and AS614). For control, the corresponding preimmune serum (*pre*) was used. *B*, PC12 cells were exposed to 100  $\mu\text{M}$  PV, 20  $\mu\text{M}$  bpV(phen), 20 mM  $\text{H}_2\text{O}_2$  (30 min each), 10 ng/ml EGF, or 50 ng/ml NGF (10 min each). Immunoprecipitates of cell lysates using anti- $\beta_1$  (AS556) were immunoblotted with anti-pY or anti- $\beta_1$  (AS614). *C*, isolated rat aortae were washed with PBS and cut into rings of 3-mm height. Slices were incubated in DMEM, 10% FCS in the absence (*control*) or presence of 100  $\mu\text{M}$  PV for 1 h. Tissues were homogenized and lysed, and immunoprecipitation was done with anti- $\beta_1$  (AS556) followed by Western blotting with anti-pY (*left*) or anti- $\alpha_1\beta_1$  (mixture of AS613 and AS614; *right*). *D*, vascular smooth muscle cells were isolated from rat aorta and incubated in DMEM, 10% FCS in the absence (*control*) or presence of 100  $\mu\text{M}$  PV for 30 min. Cell lysates were prepared, and immunoprecipitation was done with anti- $\beta_1$  (AS556) followed by Western blotting with anti-pY (*left*) or anti- $\alpha_1\beta_1$  (mixture of AS613 and AS614; *right*).

the prime role of NO in sGC activation is undisputed and the underlying activation mechanisms are well established, there is no clear understanding of the role of other, accessory mechanisms that may contribute to the fine-tuning of this key signaling enzyme. Thus, an important area of investigation in this field has been the identification and characterization of novel mechanisms contributing to the regulation of the activity, assembly, and/or localization of sGCs *in vivo*. Indeed, it has been suggested that modifications such as reversible phosphorylation could add another tier of complexity to sGC regulation. For instance some reports have documented the phosphorylation of sGC on Ser/Thr residues (19–24). Also, Tyr phosphorylation has been implicated in sGC regulation (25); however, the molecular details underlying these processes and the biological consequences of sGC phosphorylation are still obscure.

In this study we demonstrate that sGC is phosphorylated on tyrosine residue(s) in the presence of Tyr phosphatase inhibitors PV and bpV(phen). PTP inhibitor-induced tyrosine phosphorylation specifically targets the regulatory domain of the  $\beta_1$  subunit where Tyr-192 is the major, although not exclusive phosphorylation site. PTP inhibition changes sGC activity in two ways: the basal activity increases more than 2-fold, whereas the sensitivity toward NO decreases, and the corresponding  $\text{EC}_{50}$  value more than doubles, with potentially important consequences for cellular homeostasis and activity. The findings presented herein appear to match with previous observations that overexpression of SHP-1, a cytosolic PTP, inhibited basal guanylyl cyclase activity by 56% (25). However, our finding that mutant  $\beta_1$ [Y192F] displays the same enzyme kinetics as WT  $\beta_1$  clearly suggests that Tyr phosphorylation, *e.g.* of associated proteins of sGC, could underlie the observed changes in cyclase activity. Our previous observation that a multidomain protein, AGAP1, binds to sGC in a phosphorylation-dependent manner (33) may exemplify such a possibility.

Because Tyr phosphorylation of  $\beta_1$  was sensitive to PP1 and PP2, we consider Src-like kinases prime candidates for sGC kinases. This conclusion is reinforced by the fact that Tyr-192 is embedded in the highly acidic sequence of  $^{188}\text{EEDFYEDLD}$ , which resembles the phospho-ac-

ceptor sites of Vav and HS1 for p72syk kinase and matches the consensus target sequence for Src-like kinases (34, 35). Although the p72syk inhibitor piceatannol did not prevent PV-triggered Tyr-192 phosphorylation (data not shown), we could clearly demonstrate that PP1 and PP2, at concentrations where they are considered to be largely specific for Src-like kinases, curtailed Tyr phosphorylation of sGC $\beta_1$  in a dose-dependent manner. Our initial efforts to pinpoint the kinase(s) involved in sGC phosphorylation employing mouse embryo SYF and Src $^{++}$  cells revealed that Src, Yes, and Fyn are dispensable for PV-induced  $\beta_1$  phosphorylation, pointing to the possibility that another member(s) of the mammalian Src-like kinase family comprising at least 11 distinct members (41) may mediate  $\beta_1$  phosphorylation.

A crucial finding of the present study is that Tyr(P)-192 recruits Src kinase family members Src and Fyn, but not unrelated tyrosine kinase Abl or phospholipase C $\gamma$ 1, through their cognate SH2 domains. One may speculate that the initial phosphorylation of Tyr-192 drives recruitment of Src-like kinases to sGC $\beta_1$ , thereby promoting subsequent phosphorylation of other Tyr residues in  $\beta_1$ . In line with this notion we observed that Y192F mutation attenuated PV-induced phosphorylation of  $\beta_1$  and that co-expression of Src partially rescued this phenotype. Apparently the lack of a SH2 docking site at position 192 of  $\beta_1$  in the absence of  $\alpha_1$  can be overcome by high levels of active Src kinase. In contrast, Src-mediated phosphorylation was attenuated in the mutant holoenzyme, suggesting that secondary phosphorylation sites of  $\beta_1$  are not readily accessible in the  $\alpha_1\beta_1$ [Y192F] mutant and further underlining the specificity of the proposed recruitment mechanism. We also noted a minor although significant up-shift of phosphorylated WT  $\beta_1$  in the presence of Src but not of PV, consistent with the idea of multiple phosphorylation of the sGC  $\beta_1$  subunit through Src-like kinase(s). We envisage that binding of Src to Tyr(P)-192 (and subsequent phosphorylation of other Tyr residues in  $\beta_1$ ) may serve to drive incorporation of sGC into larger complexes. For instance, binding of the  $\alpha_2\beta_1$  isoform of sGC to PSD95 translocates the enzyme to the synaptic membrane and positions it in proximity to neuronal NO synthase (15). Because PSD95 also couples to *N*-methyl-D-aspartate receptors that are highly sensitive to Src phosphorylation (42, 43), one may speculate that sGC could act as an adaptor placing Src in close proximity to its target proteins.

Broad-specificity PTP inhibitors such as PV and bpV(phen) have proven useful tools to study the Tyr phosphorylation of transducer proteins; however, due to their indirect mode of action the question remains, which primary stimuli and pathways are responsible for the initial phosphorylation events. One possibility could be the activation of receptor-tyrosine kinases; however, we failed to induce Tyr phosphorylation of sGC by growth factors such as EGF or NGF, and therefore, at least some of the canonical receptor-tyrosine kinase activation pathways appear not to be involved in sGC Tyr phosphorylation. Alternatively ligand-independent transactivation of receptor-tyrosine kinases may be induced by UVC light (44–46) or ionizing irradiation (47), most likely through transiently increased production of ROS such as  $\text{H}_2\text{O}_2$ . For example,  $\text{H}_2\text{O}_2$  boosts Tyr phosphorylation-dependent signaling pathways through the activation of receptor-tyrosine kinases (27) and non-receptor tyrosine kinases such as Src (29–31, 48) or through the inhibition of PTP (44). Indeed we found that  $\text{H}_2\text{O}_2$  and to a lesser extent UVC light induces Tyr phosphorylation of sGC $\beta_1$  in intact cells. Furthermore, production of  $\text{H}_2\text{O}_2$  and superoxide by externally applied xanthine oxidase clearly induced phosphorylation of sGC $\beta_1$  although at a low level. Xanthine oxidase is expressed by vascular cells, but upon secretion it may also circulate in plasma and bind to the extracellular matrix of endothelial cells such that it can produce significant amounts of ROS under pathophysiological conditions (49). In line with our findings presented herein, Houston *et al.* (50) have shown that endotheli

um-bound xanthine oxidase inhibits NO-dependent cGMP production in smooth muscle cells. Thus, it appears that signaling cascades triggered by various stimuli converge at the level of receptor-tyrosine kinase activation and PTP inhibition.

Taken together our results demonstrate that PTP inhibitors, ROS, or UVC light may induce Tyr phosphorylation of sGC $\beta_1$  most likely through Src-like kinases *in vitro* as well as in intact PC12 cells, rat vascular smooth muscle cells, and in rat aortic tissue, pointing to the biological relevance of our findings *in vivo*. They emphasize the notion that oxidative burst events, e.g. in endothelial cells, may trigger Tyr phosphorylation of sGC in adjacent smooth muscle cells and exemplify an unexpected molecular cross-talk between tyrosine kinase and cGMP-signaling pathways.

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