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# Human substance P receptor undergoes agonist-dependent phosphorylation by G protein-coupled receptor kinase 5 in vitro

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**Abstract G protein-coupled receptor kinases (GRKs) phosphorylate agonist-occupied G protein-coupled receptors, leading to receptor desensitization. Seven GRKs, designated GRK1 through 7, have been characterized. GRK5 is negatively regulated by protein kinase C. We investigated whether human substance P receptor (hSPR) is a substrate of GRK5. We report that membrane-bound hSPR is phosphorylated by purified GRK5, and that both the rate and extent of phosphorylation increase dramatically in the presence of substance P. The phosphorylation has a high stoichiometry (20 ± 4 mol phosphate/mol hSPR) and a low $K_m$ (1.7 ± 0.1 nM). These data provide the first evidence that hSPR is a substrate of GRK5. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

*Key words:* Human substance P receptor; G protein-coupled receptor kinase; Receptor phosphorylation; Desensitization

## 1. Introduction

A wide array of physiological functions (vision, smell, taste, heart rate, neurotransmission, and cell proliferation) are regulated through the actions of G protein-coupled receptors (GPCRs) [1]. Stimulation of GPCRs by specific agonists not only transmits signals to G proteins and effectors but also modifies GPCR function by means of desensitization, sequestration, internalization, and down-regulation. According to current models, agonist-dependent phosphorylation of GPCRs plays a key role in receptor desensitization [2,3]. The agonist-dependent phosphorylation of a GPCR can be catalyzed both by second messenger-activated protein kinases, such as protein kinase A (PKA) or protein kinase C (PKC), and by members of the G protein-coupled receptor kinase (GRK) family. GRK-catalyzed phosphorylation of GPCRs facilitates binding of arrestins, which disrupt receptor/G protein coupling resulting in a loss of GPCR signaling [2,3]. Since GRKs mediate agonist-dependent phosphorylation of GPCRs, they represent an important family of enzymes in the regulation of cell signaling.

Seven distinct GRKs, termed GRK1 through GRK7, have been identified to date [4–7]; these isozymes differ in their tissue distribution, mechanism of association with the plasma membrane, and autophosphorylation [4,5,8,9]. Differences have also been reported in the regulation of GRKs by PKC and the calcium binding protein, calmodulin [5,9]. Calmodulin strongly inhibits GRK5 but only weakly inhibits GRK2. Also, PKC-catalyzed phosphorylation of GRK2 and GRK5 causes the opposite effects: GRK2 activity increases while GRK5 activity decreases [5]. This regulation of GRK activity is of particular importance in determining cellular responses. For example, since a decrease in GRK5 activity was observed after phosphorylation by PKC, GRK5 was not expected to regulate phospholipase C (PLC)-coupled receptors because stimulation of these receptors activates PKC [10].

While our understanding of the biology of various GRKs has grown considerably, the substrate specificity of most GRKs remains poorly defined [4], because the native expression level of their substrates, GPCRs, is quite low. To date, only a few GPCRs have been directly tested as GRK substrates. This situation should change soon, however, because many cloned GPCRs are being expressed at high levels in Sf9 insect cells using the baculovirus expression system [11]. Previously we reported a high level of expression of human substance P receptor (hSPR) in Sf9 cells and have demonstrated that hSPR in urea-washed membranes is efficiently phosphorylated by GRK2 [12,13]. Consistent with these in vitro findings, we have also found that hSPR stimulation leads to the rapid translocation of GRK2 from the cytosol to the plasma membrane of live cells [14]. In the present study we tested whether hSPR is a substrate of GRK5 using an in vitro approach. We show that hSPR in urea-washed Sf9 membranes undergoes agonist-dependent phosphorylation by purified GRK5. The phosphorylation of hSPR yields a high stoichiometry and the reaction is characterized by a low $K_m$. These data suggest that GRK5 may play a role in hSPR function.

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*Abbreviations:* GPCR, G protein-coupled receptor; PKA, protein kinase A; PKC, protein kinase C; GRK, G protein-coupled receptor kinase; Gβγ, G protein βγ-subunit; PLC, phospholipase C; hSPR, human substance P receptor; Sf9, *Spodoptera frugiperda*; SP, substance P; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris–HCl, Tris[hydroxymethyl]aminoethane hydrochloride); PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; AR, adrenergic receptor

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## 2. Materials and methods

### 2.1. Materials

Aprotinin, bacitracin, chymostatin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor, calmodulin, heparin, and substance P (SP) were from Sigma (St. Louis, MO, USA); [$^3$H]SR140333 (27.8 Ci/mmol) and [$^{32}$P]γ-ATP (3000 Ci/mmol) were from New England Nuclear (Boston, MA, USA). Graces insect cell medium, gentamicin, and fetal bovine serum were from Gibco (Gaithersburg, MD, USA); Sf9 cells were from Invitrogen (Carlsbad, CA, USA); diethylaminoethyl-Sephacel and heparin-Sepharose were from Pharmacia (Piscataway, NJ, USA); bovine retinas were from Pel-Freez (Rogers, AK, USA). The non-peptide SPR antagonist CP-96345 was a gift from Dr. Saul Kadin, Pfizer Inc., Groton, CT, USA. Anti-GRK5 antibody and a goat-anti-rabbit, horseradish peroxidase-conjugated antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Southern Biotechnology Associates Inc. (Birmingham, AL, USA) raised a rabbit antiserum, directed against 15 amino acids at the C-terminus of hSPR (KTMTESFSFSSNVLS); as previously reported, this hSPR antiserum is suitable for immunoblotting as well as receptor immunoprecipitation [12]. Chemiluminescence reagent (SuperSignal®) was obtained from Pierce (Rockford, IL, USA). Urea-washed Sf9 membranes expressing hSPR were prepared as previously described [12]. We used urea-washed Sf9 plasma membranes because, as we showed previously, urea-washing increases the phosphorylation of hSPR. Presumably this increase is due to the inactivation of ATP-degrading ATPases in the non-urea-washed membranes [12]. Similar to GRK2 [12], GRK5 also phosphorylated hSPR in non-urea-washed plasma membranes (data not shown). The level of hSPR in urea-washed Sf9 membranes was quantitated as previously described [13] by ligand binding using [$^3$H]SR140333. Urea-washed bovine rod outer segment membranes were also prepared as previously described [12].

### 2.2. Isolation of human GRK5 cDNA and expression in Sf9 cells

The cDNA of GRK5 was isolated from a human heart cDNA library by polymerase chain reaction using primers based on the published sequence [15]. The GRK5 clone was verified by DNA sequencing, which revealed three differences in the product relative to the published sequence: position 1059 C > T, position 1062 C > A, and position 1164 T > C. These base changes do not affect the amino acid sequence. GRK5 cDNA was cloned into the baculovirus transfer vector pVL1392 (Invitrogen, Carlsbad, CA, USA) and a high titer, recombinant baculovirus expressing GRK5 was prepared using the BaculoGold® transfection kit (PharMingen, San Diego, CA, USA). The high titer, recombinant baculovirus was used to infect Sf9 insect cells at a density of $2–2.5\times10^6$ cells/ml in spinner flasks. After 42–48 h of incubation at 27°C, cells were harvested and stored at −80°C. The maximal expression time for GRK5 was determined by immunoblotting with GRK5-specific antibody.

### 2.3. Purification of GRK5 expressed in Sf9 cells

Recombinant GRK5 was purified from Sf9 insect cell lysate following a two-step procedure adapted from a previously published method [16]. GRK5 was eluted first from an S-Sepharose column and then from a heparin-Sepharose column, unlike Kunapuli et al. [16], in which the second column was Mono-S. Unless otherwise noted, all buffers contained the following protease inhibitors: 1 mM benzamidine, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml pepstatin A, and 200 μM PMSF. Preparation of cell lysates and subsequent elution from an S-Sepharose column were similar to Kunapuli et al. [16]. Modifications include absence of 0.02% Triton from the lysis buffer, centrifugation of lysate at $100\,000\times g$, equilibration of the S-Sepharose column with 300 mM NaCl, and an elution gradient of 10 bed-volumes ranging from 300 to 800 mM NaCl at a flow rate of 0.2 bed-volumes/min. Fractions (2 ml) were collected and assayed for receptor kinase activity as previously described [12] using rhodopsin in urea-washed bovine rod outer segments as a substrate. Elution fractions containing the GRK5 activity were pooled and then applied to a 5 ml heparin-Sepharose column equilibrated with buffer A plus 250 mM NaCl. The heparin column was washed with 10 column volumes of buffer A plus 400 mM NaCl and eluted with a 100 ml linear NaCl gradient from 400 mM to 1 M in buffer A containing 0.02% Triton X-100. Fractions containing peak GRK5 activity (occurring at approximately 590–640 mM NaCl) were pooled, concentrated to approximately 500 μl using a Centriprep-30 (Millipore, Bedford, MA, USA), and then diluted 10-fold with buffer A plus 20 mM NaCl. The GRK5 preparation was then concentrated to approximately 600 μl, glycerol was added to a final concentration of 10%, and the enzyme was stored at −80°C. The purity of GRK5, as determined by silver staining, was > 95%. No contamination by GRK2 was detectable on immunoblots of up to 2.3 μg of this preparation, nor was GRK2 detected in Sf9 plasma membrane preparations (data not shown).

Characteristics of the purified GRK5 matched those previously reported for GRK5 [11,16]. Upon autophosphorylation, the GRK5 appears as a doublet with the faster-migrating (f) form converting to the slower-migrating (s) form as autophosphorylation proceeds (Fig. 3); this characteristic of GRK5 autophosphorylation has been described [10]. In individual preparations the stoichiometry of GRK5 autophosphorylation ranged from 0.2 to 1.5 pmol phosphate/pmol GRK5. Calmodulin/$Ca^{2+}$ inhibits the GRK5-catalyzed phosphorylation of rhodopsin with an $IC_{50}$ of 200 nM, and stimulates GRK5 autophosphorylation as previously reported [17].

### 2.4. In vitro phosphorylation of hSPR with purified GRK5

Receptor phosphorylation assays were performed at 30°C in a total volume of 50 μl containing 20 mM Tris–HCl (pH 7.4), 2 mM EDTA, 4 mM $MgCl_2$, and 0.1 μg of purified GRK5, plus different amounts of Sf9 membrane proteins to vary the concentration of hSPR in the reaction. Other components are noted in the figure legends. Reactions were started by adding 5 μl of [$^{32}$P]γ-ATP (1.0–1.3 mM; specific activity, 2–3 cpm/fmol) and stopped by adding 25 μl of sodium dodecyl sulfate (SDS) sample buffer (0.06 M Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.025% bromophenol blue and 10% freshly added 2-mercaptoethanol). Phosphorylated receptors were visualized by subjecting the samples to 10% SDS–polyacrylamide gel electrophoresis (PAGE) followed by autoradiography. The phosphorylated receptor bands were excised from the dried gels, counted in a scintillation counter, and the stoichiometry of phosphorylation was calculated from the amount of receptor loaded onto the gel and the specific activity of [$^{32}$P]γ-ATP. As we have reported previously [12], increasing the concentration of hSPR reduces the stoichiometry of phosphorylation. Accordingly, the hSPR concentration was held to a low level ($\approx$1 nM) for determining the phosphorylation stoichiometry. For kinetic studies, the concentration of membrane-bound hSPR varied from 1 to 60 nM, and initial rates were determined by stopping the reactions after 3 min; the reaction rate was linear up to 6 min at the lowest receptor concentration (1 nM). The $K_m$ and $V_{max}$ values were obtained by a non-linear fit of the data to the Michaelis–Menten equation using the computer program, KaleidaGraph™ (Synergy Software, Reading, PA, USA).

## 3. Results

### 3.1. Phosphorylation of hSPR in Sf9 membranes with purified GRK5

Fig. 1 shows phosphorylation of hSPR in urea-washed Sf9 membranes by purified GRK5 in the absence or presence of SPR ligands. As can be seen in Fig. 1 (lane 2), there is a marked increase in the phosphorylation of a 45 kDa protein upon addition of SP. Immunoprecipitation by hSPR-specific antiserum identifies the 45 kDa protein as phosphorylated hSPR (data not shown). The SP-dependent phosphorylation of hSPR by GRK5 is blocked by the SPR antagonist, CP-96345 (Fig. 1, lane 3). These results indicate that hSPR phosphorylation by GRK5 is triggered by activation of hSPR rather than occupancy of the ligand-binding site.

### 3.2. Characterization of GRK5-catalyzed phosphorylation of SPR

The GRK5-catalyzed phosphorylation of hSPR is dependent on the concentration of SP, and half-maximal phosphorylation ($EC_{50}$) occurs at $31\pm2$ nM SP (Fig. 2). This value reflects binding of SP to the low-affinity state of hSPR [18],

and is similar to the $EC_{50}$ for SP-dependent phosphorylation of hSPR by GRK2 [12].

Fig. 3 shows a time-course of GRK5-catalyzed phosphorylation of hSPR in the absence and presence of SP. In the absence of SP, GRK5 phosphorylates hSPR slowly to a maximal stoichiometry of 4.0 ± 0.8 mol phosphate/mol receptor, which increases to 20 ± 4 mol phosphate/mol receptor following stimulation with SP. The stoichiometry of GRK5-catalyzed hSPR phosphorylation is comparable to the stoichiometry of 19 ± 1 mol phosphate/mol receptor we reported previously for GRK2-catalyzed phosphorylation of agonist-occupied hSPR [12].

### 3.3. Kinetics of hSPR phosphorylation by GRK5

Fig. 4 shows initial rates of GRK5-catalyzed phosphorylation of agonist-occupied hSPR as a function of increasing

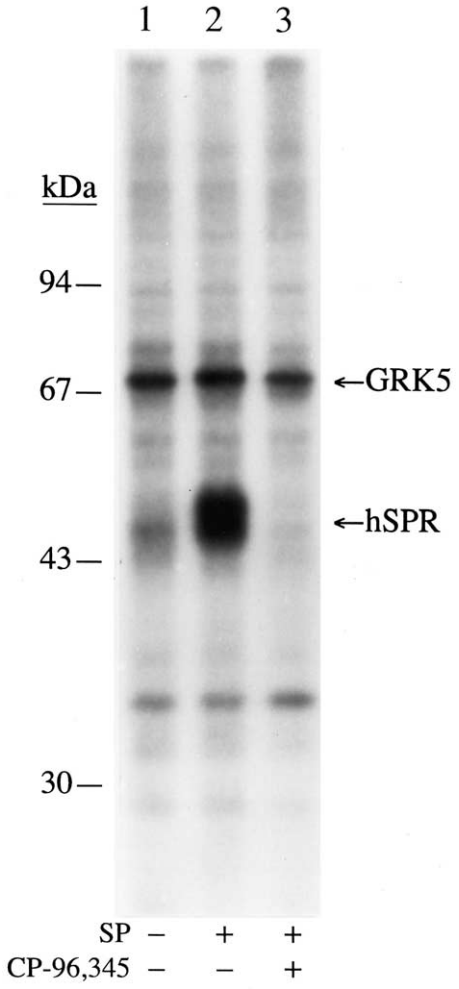



Fig. 1. GRK5-catalyzed phosphorylation of hSPR in urea-washed Sf9 membranes. Receptor phosphorylation was performed in a 50 μl volume containing 70 fmol of hSPR in urea-washed Sf9 membranes with a specific activity of 140 pmol hSPR/mg protein, 30 nM GRK5, 0.12 mM [$^{32}$P]γ-ATP (2–3 cpm/fmol), ± 1 μM SP, ± 500 μM SPR antagonist CP-96,345, 20 mM Tris–HCl (pH 7.4), 2 mM EDTA, and 4 mM $MgCl_2$. Reactions proceeded for 60 min at 30°C and were stopped with 25 μl SDS sample buffer, then analyzed by SDS–PAGE/autoradiography. Background phosphorylation, without GRK5 but with SP, was less than 1% (data not shown). The experiment was repeated three times.

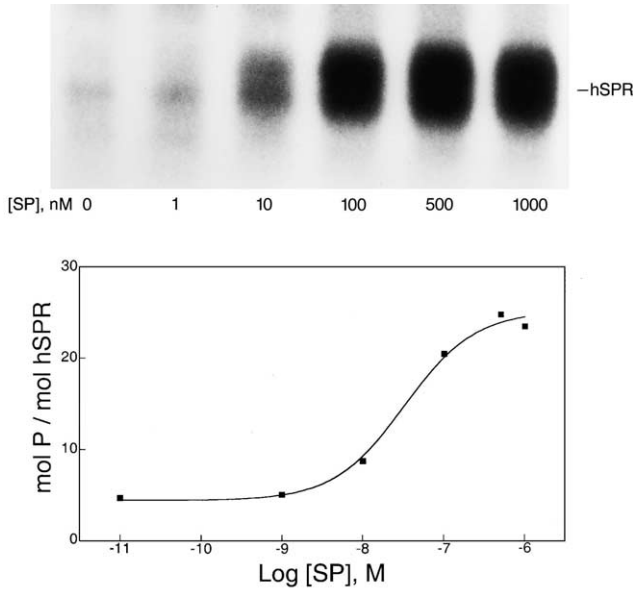



Fig. 2. Effect of increasing SP concentration on hSPR phosphorylation by GRK5. Phosphorylation reactions were performed in a 50 μl volume containing 50 fmol hSPR in urea-washed Sf9 membranes with a specific activity of 100 pmol of hSPR/mg membrane protein, 30 nM GRK5, 0.12 mM [$^{32}$P]γ-ATP (2–3 cpm/fmol), 20 mM Tris–HCl (pH 7.4), 2 mM EDTA, and 4 mM $MgCl_2$. The reaction proceeded for 60 min at 30°C and was stopped by adding 25 μl SDS sample buffer, then analyzed by SDS–PAGE/autoradiography. The experiment was repeated three times.

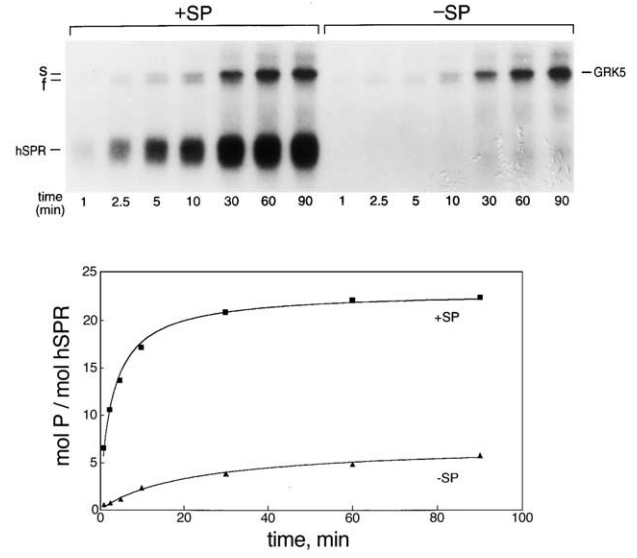



Fig. 3. Time-course of hSPR phosphorylation by GRK5 in urea-washed Sf9 membranes. Phosphorylation reactions were performed in a 50 μl volume containing 70 fmol of hSPR in urea-washed membranes with a specific activity of 190 pmol of hSPR/mg protein, 30 nM GRK5, 1 μM SP, 0.12 mM [$^{32}$P]γ-ATP (2–3 cpm/fmol), 20 mM Tris–HCl (pH 7.4), 2 mM EDTA, and 4 mM $MgCl_2$. The reaction mixtures were incubated at 30°C for the indicated times, stopped by adding 25 μl of SDS sample buffer, and then analyzed by SDS–PAGE/autoradiography. After autoradiography, the receptor bands were excised, counted, and the stoichiometry of phosphorylation was determined. The experiment was repeated twice.

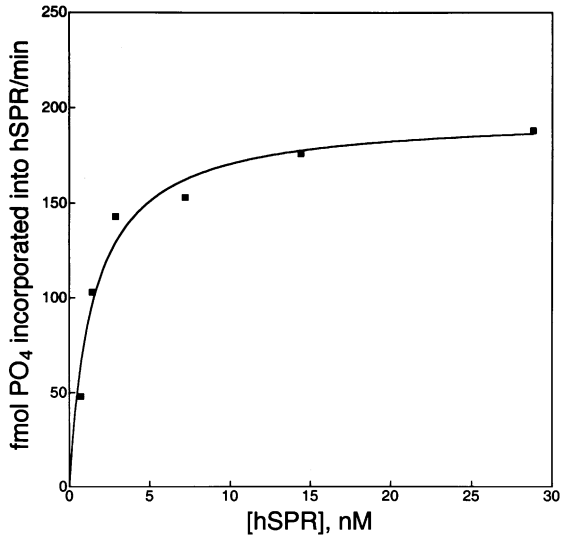



Fig. 4. Kinetic analysis of GRK5-catalyzed phosphorylation of agonist-occupied hSPR in Sf9 membranes. Phosphorylation reactions were performed in a 50 μl volume containing approximately 0.5–25 nM hSPR in urea-washed Sf9 membranes (specific activity: 160 pmol hSPR/mg membrane protein), 3–7 nM GRK5, 1 μM SP, 0.12 mM [$^{32}$P]γ-ATP (2–3 cpm/fmol), 20 mM Tris–HCl, (pH 7.4), 2 mM EDTA, and 4 mM $MgCl_2$. Incubations were stopped after 3 min at 30°C by adding 25 μl SDS sample buffer. Receptor phosphorylation was visualized by SDS–PAGE/autoradiography. Initial rates were determined by excising receptor band and counting radioactivity. Data points represent mean values of three separate experiments performed in duplicate. Data were fitted to the Michaelis–Menten equation using KaleidaGraph™ (Synergy Software, Reading, PA, USA).

concentrations of membrane-bound hSPR. Analysis of these data according to the Michaelis–Menten equation shows that the reaction occurs with a $K_m$ of 1.7 ± 0.1 nM and a $V_{max}$ of 10 ± 3 nmol/min/mg (S.E.M. $n = 5$). Under similar conditions, rhodopsin is phosphorylated by GRK5 with a $K_m$ of 5 μM; this is similar to the $K_m$ values of 2–16 μM reported by other investigators [16,19] for GRK5-catalyzed rhodopsin phosphorylation. The $K_m$ of 1.7 nM for GRK5-catalyzed phosphorylation of hSPR is comparable to the $K_m$ of 3.6 nM obtained for GRK2-catalyzed phosphorylation of hSPR in the presence of Gβγ [12].

## 4. Discussion

This study provides the first demonstration that hSPR undergoes agonist-dependent phosphorylation catalyzed by GRK5 in vitro. The GRK5-catalyzed phosphorylation of hSPR occurs with a high stoichiometry (20 mol phosphate/mol receptor) and a low $K_m$ (1.7 nM). These characteristics of GRK5-catalyzed phosphorylation of hSPR are very similar to the characteristics of in vitro phosphorylation of hSPR by GRK2 [12]. Thus, hSPR is a good substrate of both GRK5 and GRK2. In the case of GRK2, the high affinity observed for hSPR in our in vitro studies was substantiated by the in vivo data in which we demonstrated a sustained translocation of GRK2 to plasma membrane upon activation of hSPR in live cells [14]. Whether GRK5 exhibits high affinity for hSPR in vivo remains to be seen.

The main finding of our study is that hSPR is efficiently phosphorylated by GRK5 in vitro. Previous in vitro studies have tested the ability of GRK5 to phosphorylate GPCRs that exert their actions through Gαs ($\beta_2$-adrenergic receptor (AR)) or $G\alpha_i$ (m2 muscarinic and $\alpha_2$C2-AR) pathways. These studies found that GRK5 phosphorylates $\beta_2$-AR efficiently [16,19] but phosphorylates m2-muscarinic and $\alpha_2$C2-ARs poorly [20,21]. The present study extends these observations by showing that GRK5 efficiently phosphorylates hSPR, which couples with $G_q$ and stimulates PLC [22]. The ability of GRK5 to phosphorylate hSPR is of interest because it has been found previously that phosphorylation of GRK5 by PKC causes a five-fold increase in the $K_m$ and a 2.8-fold decrease in the $V_{max}$ of rhodopsin phosphorylation by GRK5 [10]. Thus GRK5 would not be predicted to act on the PLC-coupled receptor. Consistent with this expectation, GRK5 does not significantly increase agonist-dependent phosphorylation of either the angiotensin II type 1 receptor [23] or the $\alpha_{1b}$-AR [24], both of which stimulate PLC. Furthermore, transgenic mice over-expressing GRK5 have been found to have a marked desensitization of β-adrenergic response, but the contractile response to angiotensin II, which functions through $G_q$-coupled angiotensin receptors, remains unchanged [25]. Clearly, these findings do not indicate any interaction between GRK5 and PLC-coupled GPCRs. However, more recent evidence supports a significant role for GRK5 in the regulation of PLC-coupled receptors such as angiotensin II type 1A [26], metabotropic glutamate receptor 1A [27], $V_1$-vasopressin receptor [28], and thrombin receptor [29]. Furthermore, recent data from hybrid transgenic mice over-expressing a constitutively active mutant of $\alpha_{1b}$-AR and GRK5 indicate GRK5 is similarly involved in the regulation of $\alpha_{1b}$-AR [30]. These data, taken together with our demonstration of a high affinity interaction between GRK5 and hSPR, support the notion that GRK5 can regulate the function of PLC-coupled receptors.

Our results indicate that GRK5, like GRK2 [12], phosphorylates hSPR to a high stoichiometry. Thus, it is likely that the majority of hSPR's 26 C-terminal serine/threonine residues and 12 serine/threonine residues on the intracellular loops become phosphorylated by GRK2 and GRK5. In this respect, hSPR is like β2-AR which also undergoes robust phosphorylation by both GRK2 and GRK5, and these two isozymes phosphorylate β2-AR on overlapping sites [31]. Our own preliminary data from two-dimensional phosphopeptide maps of hSPR following in vitro phosphorylation by either GRK2 or GRK5 show 10 distinct phosphopeptides, most of which are common to both GRK2 and GRK5 (unpublished observation). The physiological significance of these findings also remains to be evaluated. It should be pointed out that hSPR is not the only GPCR whose phosphorylation by a GRK occurs with a high stoichiometry. For example, $\beta_2$-AR reconstituted into phospholipid vesicles is phosphorylated by GRK2 to a stoichiometry of 4 mol phosphate/mol of receptor which increases to 11 mol of phosphate/mol receptor in the presence of Gβγ [32]. Similarly, m2-muscarinic receptor is phosphorylated by GRK2 to a stoichiometry of 4–5 mol phosphate/mol receptor, which increases two- to three-fold in the presence of Gβγ [33].

In summary, we provide the first evidence that GRK5 efficiently phosphorylates hSPR in an agonist-dependent manner. Future studies will determine whether GRK5 plays a role in the function of hSPR in vivo.

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