β_2-Adrenergic Receptor Stimulated, G Protein-Coupled Receptor Kinase 2 Mediated, Phosphorylation of Ribosomal Protein P2[†]

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ABSTRACT: G protein-coupled receptor kinases are well characterized for their ability to phosphorylate and desensitize G protein-coupled receptors (GPCRs). In addition to phosphorylating the β_2-adrenergic receptor (β_2AR) and other receptors, G protein-coupled receptor kinase 2 (GRK2) can also phosphorylate tubulin, a nonreceptor substrate. To identify novel nonreceptor substrates of GRK2, we used two-dimensional gel electrophoresis to find cellular proteins that were phosphorylated upon agonist-stimulation of the β_2AR in a GRK2-dependent manner. The ribosomal protein P2 was identified as an endogenous HEK-293 cell protein whose phosphorylation was increased following agonist stimulation of the β_2AR under conditions where tyrosine kinases, PKC and PKA, were inhibited. P2 along with its other family members, P0 and P1, constitutes a part of the elongation factor-binding site connected to the GTPase center in the 60S ribosomal subunit. Phosphorylation of P2 is known to regulate protein synthesis in vitro. Further, P2 and P1 are shown to be good in vitro substrates for GRK2 with K_M values approximating 1 μM. The phosphorylation sites in GRK2-phosphorylated P2 are identified (S102 and S105) and are identical to the sites known to regulate P2 activity. When the 60S subunit deprived of endogenous P1 and P2 is reconstituted with GRK2-phosphorylated P2 and unphosphorylated P1, translational activity is greatly enhanced. These findings suggest a previously unrecognized relationship between GPCR activation and the translational control of gene expression mediated by GRK2 activation and P2 phosphorylation and represent a potential novel signaling pathway responsible for P2 phosphorylation in mammals.

The agonist-occupied G protein-coupled receptors (GPCRs)[1] are specifically phosphorylated by a family of serine/threonine kinases (GRKs) that thereby initiate their desensitization. GRK2, the most thoroughly characterized member of this family of kinases, is expressed in a wide variety of tissues and phosphorylates many known GPCRs including adrenergic receptors. GRK activity is tightly regulated by heterotrimeric G protein $\beta\gamma$, lipids, calcium-binding proteins, cytoskeletal proteins, and protein kinases (*1*−*4*). Interestingly, agonist-occupied receptors also allosterically activate GRKs. GRK-mediated peptide phosphorylation is greatly enhanced in the presence of activated receptors (*1*). Therefore, following agonist stimulation, receptors whose signal is dampened by GRKs could in turn activate the GRK-mediated phosphorylation of nonreceptor substrates, thus triggering novel GPCR signaling pathways. The identification of tubulin (*2*, *4*, *5*), synucleins (*6*), and phosducin (*7*) as nonreceptor substrates of GRK2 provides evidence that indeed nonreceptor substrates exist for GRKs. Further, the phosphorylation of tubulin is increased following agonist stimulation, providing additional evidence to support a role of GPCRs in the activation of GRKs (*2*).

Here we identify the 60S ribosomal protein P2 as a novel substrate of β_2-adrenergic agonist stimulated GRK2. P2 has previously been demonstrated to be one of the few ribosomal proteins that can be phosphorylated in vivo and in vitro, although the kinase(s) responsible for its phosphorylation in higher eukaryotes is (are) not fully characterized (*8*−*10*). We demonstrate that the GRK2-mediated phosphorylation of P2 takes place on the sites previously localized for another

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[1] Abbreviations: GRK, G protein-coupled receptor kinase; GPCR, G protein-coupled receptor; β_2AR, β_2-adrenergic receptor; G$\beta\gamma$, G protein subunits $\beta\gamma$; CK2, casein kinase 2; eEF-2, eukaryotic elongation factor-2; eIF-2α, eukaryotic initiation factor-2α; Iso, isoproterenol; P1, 60S ribosomal protein P1; P2, 60S ribosomal protein P2; DMMA, 2,3-dimethylmaleic anhydride; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; MOWSE, molecular weight search.

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kinase, casein kinase 2 (CK2) (*11*). Moreover, we confirm that this phosphorylation at the C-terminal part of P2 leads to a functional activation of the ribosome (*12*), which suggests that GRK2 activation might be involved in the regulation of protein synthesis.

EXPERIMENTAL PROCEDURES

Materials. Tissue culture media and fetal bovine serum (FBS) were obtained from Life Technologies (Rockville, MD). Isoproterenol and ICI-118,551 were obtained from Sigma. 2D gel electrophoresis reagents and silver stain kit were obtained from Genomic Solutions; radiolabeled orthophosphate was obtained from Dupont NEN. Herbimycin A and staurosporine were obtained from Roche Diagnostics. GRK2 was purified as previously described (*13*). Preparation of rat liver eEF-2 (95% pure), of 60S and 40S ribosomal subunits by zonal centrifugation, and of recombinant ribosomal proteins P1 and P2 has already been described (*14*, *15*). Phosphatidylcholine and phosphatidylinositol 4,5-diphosphate were from Sigma.

Cellular Preparation. HEK-293 cells were maintained in MEM supplemented with 10% FBS, 100 units/mL penicillin G, and 100 μg/mL streptomycin sulfate at 37 °C, 5% CO_2. Cells at 60−80% confluency were transfected with 11 μg of DNA, using Flag-β_2AR (1 μg) with either pRK5-GRK2 (10 μg) or empty vector (10 μg). Following transfection, cells were split into 150 mm collagen-plated dishes and allowed to attach overnight. The cells were incubated 16 h in serum-free MEM (to reduce basal GPCR activity), 10 mM Hepes, pH 7.4, 100 units/mL penicillin G, and 100 μg/mL streptomycin sulfate, and 1 μM herbimycin A (to inhibit tyrosine kinases). Cells were then rinsed in phosphate-free MEM 2 times and incubated with phosphate-free MEM containing [^{32}P]-orthophosphate (2.5 mCi/150 mm dish) and 1 μM staurosporine (to inhibit PKA and PKC) for 1 h. Cells were incubated with either 10 mM ICI-118,551 (a β_2AR inverse agonist) or Iso (a β_2AR agonist) for 5 min at 37 °C. The cells were then rinsed 3 times with ice-cold wash buffer (2.7 mM KCl, 1.5 mM KH_2PO_4, 136 mM NaCl, and 8.1 mM Na_2HPO_4). The cells were resuspended in 0.3% SDS, 200 mM DTT, 28 mM Tris-HCl, and 22 mM Tris base and incubated for 10 min at 100 °C. The samples were then incubated on ice for 5 min before a further 10 min incubation in 0.1 volume of 24 mM Tris base, 476 mM Tris-HCl, 50 mM $MgCl_2$, 1 mg/mL DNase I, and 0.25 mg/mL RNase A. Acetone was added to 80%, and proteins were centrifuged. Pellets were resuspended in 7.92 M urea, 0.06% SDS, 1.76% Ampholytes pH 3−10, 120 mM DTT, 3.2% Triton X-100, 22.4 mM Tris-HCl, 17.6 mM Tris base (sample buffer mix, Genomic Solutions).

2D Gel Electrophoresis and Mass Spectrometry of the Differentially Labeled Spots. Soluble cell extracts from one 150 mm dish were applied to a 2 mm preparatory capillary isoelectric focusing gel pH 3−10, and proteins were separated according to their isoelectric point under the following conditions: 600 μA/gel for a total of 17 500 V × h. The gels were extracted and incubated in 0.3 M Tris base, 0.075 M Tris-HCl, 3.0% SDS, 500 mM DTT, 0.01% bromophenol blue for 15 min, and applied to a 22 cm × 22 cm × 1 mm 10% SDS−PAGE resolving gel. Following electrophoresis, the gels were fixed and stained using a nondestructive silver stain (by omitting the glutaraldehyde from the Genomic Solutions Silver stain kit) and dried on cellulose (BioRad). Autorads were made from a 17 h exposure to the gels. Protein spots that were differentially phosphorylated were excised from the dried gel. A sample of gel with no protein was used as a control. The gel samples were prepared and analyzed by PE Biosystems Contract Services using MALDI-TOF (matrix-assisted laser desorption ionization time of flight) to obtain mass spectra.

Phosphorylation of Recombinant P1 and P2 Proteins. For the reaction shown in Figure 3, GRK2 phosphorylation of P-proteins was assayed as previously described for casein (*3*).

To determine the effect of lipid and G$\beta\gamma$ upon P-protein phosphorylation, phospholipid liposomes (25 mg/mL) were prepared by sonicating phosphatidylcholine and phosphatidylinositol 4,5-diphosphate (95%/5% by mass) in buffer A (40 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 10 mM $MgCl_2$, 1 mM dithiothreitol) on ice 4 times for 20 s. The ability of phospholipids and/or G$\beta\gamma$ to modulate the GRK2-mediated phosphorylation of P2 was studied by comparing incorporation of ^{32}P in P2 in the presence of these effectors to that obtained in the standard conditions (GRK2 and P2 only). A 16 μM sample of recombinant rat P2 was incubated in buffer A for 60 min at 30 °C with 40 nM human recombinant GRK2 and 500 μM ATP (100 cpm/pmol) in the presence or absence of 1 μg/μL liposomes and/or 100 nM G$\beta\gamma$ in 25 μL. Half of the incubate was run on SDS−PAGE (15%) and exposed to a Phosphorimager after Coomassie Blue staining. ^{32}P incorporation in P2 was quantitated using the Image Quant software from Molecular Dynamics.

For preparing fully biphosphorylated P2 (>95% biphosphorylated) for the determination of the sites phosphorylated by the GRK2 (Table 2), 1 mg of recombinant protein P2 was phosphorylated overnight by GRK2 (enzyme/substrate, 1:60, by mass) at 37 °C in 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 20 mM $MgCl_2$, 1 mM dithiothreitol, 2% (w/v) glycerol, and 5 mM ATP. After phosphorylation, 10% of the incubation mixture was dialyzed against 0.1% trifluoroacetic acid/water, lyophilized, and resuspended in water/methanol/formic acid (49:50:1, v/v), and the level of phosphorylation of P2 was established by electrospray mass spectrometry (ES/MS) as described below.

Determination of the Sites Phosphorylated by the GRK2 in P2. These experiments were performed using an Applied Biosystems API 165 quadrupole mass spectrometer. GRK2-phosphorylated P2 was dialyzed against water, lyophilized, and resuspended in a methanol/water/formic acid mixture (50:49.9:0.1, v/v). P2 was proved to be over 95% biphosphorylated by direct infusion of the reactional mixture in a microspray source (0.2 μL/min flow rate) operated in the positive ion mode. Calculated reconstruct mass was 13 267.9 ± 1.6 Da, which corresponds to native poly(His)-tagged P2 (13 090.5 Da) plus two phosphates and an oxidation (result not shown). GRK2-biphosphorylated P2 was then submitted to limited proteolysis with the V8 endoprotease (Glu-C) of *Staphylococcus aureus* as (*16*). The fragments containing phosphate groups were determined by ES/MS-coupled HPLC. Fragments were separated by an Applied Biosystems ABI 140D capillary LC system using a C18-HPLC column (LC Packings, 100 mm × 0.3 mm, 5 μm particle size; 300

Å pore) at a flow rate of 5 μL/min. Elution was operated in four steps by increasing the percentage of acetonitrile in water/trifluoroacetic acid (0.05%) as indicated: 10 min at 10%, 10−70% in 60 min, 70−90% in 10 min, 10 min at 90%. The column was connected to both a 785A absorbance detector set at 214 nm and an ionspray source operating either in a positive ion mode or in a negative ion mode [orifice tension values set at 40 and 65 V and ion masses (*m*/*z*) registered between 400 and 1900].

Selective Deproteinization and Reconstitution of 60S Ribosomal Subunit. DMMA (2,3-dimethylmaleic anhydride) core-particles were prepared from 60S ribosomal subunits as described previously (*15*). Reconstitution of the DMMA-core was carried out using the extracted proteins or combinations of recombinant P1 and P2 or GRK2- or CK2-phosphorylated P2 as described previously (*12*).

RESULTS

Identification of Kinase Substrates following Agonist Exposure in Vivo. To identify nonreceptor soluble GRK substrates, we developed a cellular assay utilizing 2D gel electrophoresis and mass spectrometry. To activate GRK2, cells overexpressing β_2AR, a prototypical G protein-coupled receptor, and GRK2 were stimulated with agonist (isoproterenol). The exposure of cells to agonist causes phosphorylation of numerous cellular proteins, even in the presence of herbimycin A and staurosporine which inhibit tyrosine and other serine/threonine kinases (e.g., PKA and PKC). We focused exclusively on cellular proteins whose agonist-dependent phosphorylation was enhanced in the presence of overexpressed GRK2. Cells overexpressing β_2AR and GRK2 were labeled with [^{32}P]orthophosphate, stimulated with an inverse β_2AR agonist (upper panel) or β_2AR agonist (lower panel, '+ISO'), solubilized, and then subjected to two-dimensional gel electrophoresis and autoradiography (Figure 1). Comparison of the two autoradiographs demonstrates that numerous phosphoproteins are relatively unchanged following agonist exposure (for reference, compare spots A−E). However, close observation reveals several spots that are differentially phosphorylated. Although several spots increased in intensity following agonist treatment, we focused on spot F, which gave the most robust signal. Spot F corresponds to a protein whose phosphorylation state is dependent on the expression of GRK2, with no significant phosphorylation seen in the absence of GRK2 overexpression even when stimulated (Figure 1, inset to lower panel). Spot F is a small, acidic protein with a p*I* of approximately 4 and an apparent molecular mass of approximately 17−18 kDa. To identify this protein, spot F was eluted from the gel and subjected to MALDI-TOF protein identification. The five masses obtained from the mass spectrometry procedure (Table 1, middle column) were compared with those in the Swiss Prot or NCBI database using Protein Prospector from UCSF. The masses matched with the theoretical masses from the 60S ribosomal protein P2 [Swiss Prot Accession Number P05387] (Table 1, right column) to give a high confidence MOWSE score of 50 700. The mass spectrometry identification was consistent with both the isoelectric point and the apparent molecular mass observed on the 2D gel electrophoresis (Table 1). These peptide sequences (in boldface, Figure 2) cover 47% of the sequence of P2. Interestingly, P2 is a member of the ribosomal P-protein family consisting of three members with P0 and P1. The P-proteins share highly homologous C-terminal domains that are known to be phosphorylatable in vivo, and P2 phosphorylation was shown to be required for P2 activity in translational activity (*12*) (Figure 2, phosphorylation sites are underlined). How-

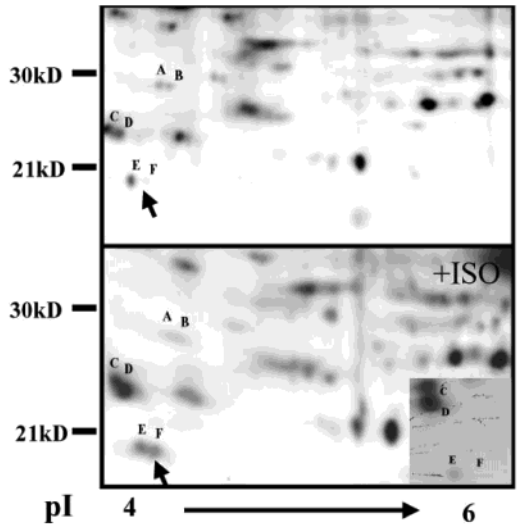

FIGURE 1: Two-dimensional gel electrophoresis of phosphoproteins in HEK-293 cells overexpressing the β_2AR and GRK2. Soluble cellular extracts were prepared and subjected to two-dimensional gel electrophoresis, and the gels were exposed to autoradiography as described under Experimental Procedures. The upper panel represents a portion of the autoradiogram displaying the soluble cellular phosphoproteins from cells overexpressing β_2AR and GRK2 following treatment with ICI-118,551 (10 μM), and the lower panel represents the matching portion from cells treated with 10 μM isoproterenol. The inset in the lower panel demonstrates cells overexpressing only β_2AR that are treated with 10 μM isoproterenol. The molecular mass standards are shown to the left of the gels, and the p*I* range increases from left to right from 4 to 6. Spots A−E are reference matching spots. Spot F (above the arrow) phosphorylation increases dramatically following isoproterenol stimulation. This experiment was repeated twice with comparable results.

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P1   MASVSE--LA CI-------Y SAL--ILHDD EVTVTEDKIN ALIKAAGVNV 39
P2  MRYVASYLLA ALGGNSSPSA KDIKKILDSV GIEADDDRLN KVI--SELNG 48

P1  EPFWPGLFAK ALANVNIGSL ICNVGAGGPA PAAGAAPAGG PAPSTAAAPA 89
P2  KNI-EDVIAQ GIG--KLASV PAGGAVAVSA APGSAAPAAG SAPAAA---- 91

P1  EEKKVEAKKE ESEESDDDMG FGLFD 114
P2  EEKK DEKKE ESEESDDDMG FGLFD 115
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FIGURE 2: Sequence comparison of human P1 and P2. Sequences corresponding to those identified by mass spectrometry (Table 1) are highlighted in boldface type. Ser 102 and Ser 105, known sites of phosphorylation, are underlined.

Table 1: Comparison of Spot F Identified by 2D-Gel Electrophoresis and 60S Ribosomal Protein P2[a]

	spot F	60S ribosomal protein P2
p*I*	~4	4.49
molecular mass	15−20 kDa	calculated 11.6 kDa apparent 17 kDa[b]
peptide masses	1256.6750	1256.6850
	1417.6820	1417.6811
	1772.9170	1772.9030
	1869.0060	1868.9758
	2156.1100	2156.1174

[a] The isoelectric point and molecular mass of spot 1 were estimated from two-dimensional gel electrophoresis. Peptide masses were obtained from MALDI-TOF analysis (see Experimental Procedures). Values for P2 were taken from public databases. [b]The difference between the apparent and the real molecular mass of P2 was already described (*12*).

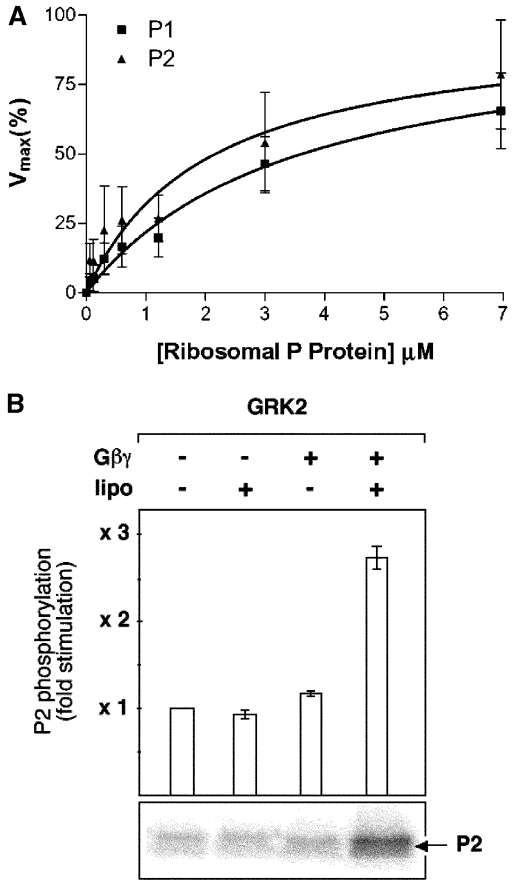

FIGURE 3: Analysis of GRK2-mediated phosphorylation of P-proteins in vivo. (A) Kinetic analysis of GRK2-mediated phosphorylation of P1 and P2. The indicated concentrations of P1 (closed squares) and P2 (closed triangles) were incubated with 0.4 μM GRK2 for 10 min at 30 °C as described under Experimental Procedures. Kinase reactions were separated by SDS−PAGE, and the gels were exposed to the Phosphorimager (Molecular Dynamics). The phosphorylated proteins were quantified, and the mean and SEM for four experiments are shown. (B) Effect of liposomes (lipo) and heterotrimeric G-proteins $\beta\gamma$ (G$\beta\gamma$) on GRK2-mediated P2 phosphorylation. 16 μM recombinant rat P2 was phosphorylated by 40 nM human recombinant GRK2 in the presence of 1 μg/μL liposomes of phosphatidylcholine and phosphatidylinositol 4,5-diphosphate [95:5 (w/w)] and/or 0.1 μM G$\beta\gamma$ as indicated. Proteins were separated on a 15% SDS−polyacrylamide gel and visualized by Coomassie blue staining. ^{32}P incorporation in P2 was visualized by exposition to a Phosphorimager (lower panel) and quantitated with the Imagequant software (upper panel). Results are the mean and SEM of three experiments.

ever, the identity of the kinase(s) responsible for this phosphorylation in vivo has not been clearly established yet (*17*).

Evaluation of GRK2 Ability To Phosphorylate P2 in Vitro. To demonstrate that GRK2-mediated phosphorylation of P2 and its family member P1 display reasonable kinetic parameters, we incubated purified GRK2 with increasing concentrations of purified recombinant ratP1 and P2. The K_Ms for the phosphorylation of P1 and P2 by GRK2 were determined to be 1.0 ± 0.1 and 0.9 ± 0.0 μM, respectively (Figure 3). This compares well with the K_M reported for tubulin (approximately 1.0 μM) (*2*), another nonreceptor substrate for GRKs, further supporting the idea that these ribosomal proteins are good substrates for GRKs. Interestingly, although several radiolabeled proteins surrounding P2 were identified by mass spectroscopy, P1 was not identified as a protein phosphorylated in the in vivo phosphorylation experiments (Figure 1). Future studies are necessary to establish whether P1 is also phosphorylated in cells overexpressing GRK2 in an agonist-dependent manner.

To further establish P2 as a novel substrate of GRK2, we assayed GRK2-mediated phosphorylation in the presence of G$\beta\gamma$ and lipids. The addition of G$\beta\gamma$ and lipid vesicles consisting of 95% PC and 5% PIP2 enhanced the GRK2-mediated phosphorylation of P2 by approximately 3-fold while addition of either G$\beta\gamma$ or lipid vesicles alone did not significantly alter the phosphorylation (Figure 3B). The increase in phosphorlation upon addition of G$\beta\gamma$ and lipid is similar to that demonstrated for other GRK2 substrates, including β_2AR, tubulin, and synuclein (*2*, *6*).

Identification of the Sites Phosphorylated by the GRK2 in P2. It has been reported that CK2 phosphorylates the C-terminus of P2 at S102 and S105 (*11*). Both GRK2 and CK2 preferentially phosphorylate serines surrounded by acidic residues, a motif found in the C-termini of P-proteins (*18*, *19*). Therefore, we postulated that GRK2 and CK2 would phosphorylate identical sites in P2. To confirm this hypothesis, we performed an overnight GRK2-mediated phosphorylation of recombinant P2 and showed by ES/MS that P2 did contain two phosphate residues as already shown for CK2-phosphorylated P2. Ions corresponding to monophosphorylated and unphosphorylated P2 were also found but should account for less than 5% of the total P2 (data not shown). Then, phosphorylated P2 was digested by the *Staphylococcus aureus* V8 protease, and P2 fragments were separated and identified by ES/MS-coupled HPLC (Figure 4A, Table 2). Ion masses were attributed to fragments of P2 delineated by the V8 protease specific cleavage sites (Glu-C and to a lower extent Asp-C) using the ProMac software. As shown in Figure 4B and Table 2, fragments corresponding to the possible cleavage sites were all found. The only fragments found as phosphorylated were those containing the C-terminal serines (S114 and S117, underlined in Figure 4B, and corresponding to S102 and S105, respectively, in the untagged protein). A closer analysis of the data indicates that the shorter biphosphorylated fragment is the 110−118 one (KKEESEESD) in the 45.4 min peak of Figure 4A. This fragment contains as the only phosphorylatable residues the two previously quoted serines. Fragment 116−127 in peak 47.8 harbors one phosphate on its only serine. The facts that no mass matching with phosphorylated fragment of other parts of P2 was identified and that many overlapping fragments comprising the other serine residues of P2 were identified give a high confidence that only the carboxy-terminal serines were able to be phopshorylated by GRK2. It is noteworthy that several ions corresponding to unphosphorylated or monophosphorylated forms of phosphorylatable C-terminal fragments were also found (fragments 114−127 and 117−127 in peak 45.4 and fragment 110−116 in peak 47.1). This is not surprising since biphosphorylated P2 had not been purified from unphosphosphorylated and monophosphorylated P2 before the V8 cleavage. Peak 47.1 contained a monophosphorylated fragment (110−127), but the serine phosphorylated first by the GRK2 cannot be determined from these data.

Reconstitution of Active 60S Ribosomal Subunits Using GRK2-Phosphorylated Recombinant P2. Phosphorylation of P2 by CK2 has been previously demonstrated to stimulate

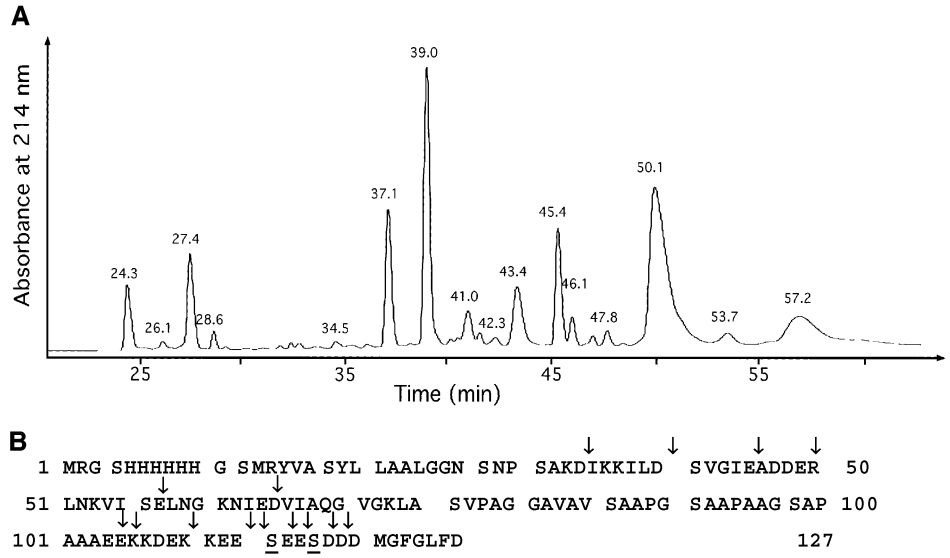

FIGURE 4: Determination of the GRK2 phosphorylation sites in P2. (A) HPLC profile of the GRK2-biphosphorylated rat P2 V8 fragments registered at 214 nm. P2, extensively phosphorylated by GRK2, was found to be biphosphorylated (over 95%) by electrospray/mass spectrometry (ES/MS) (result not shown). Then, P2 was proteolyzed by the V8 endoprotease (Glu-C) of *Staphylococcus aureus*, and fragment separation and identification was performed by ES/MS-coupled HPLC as described under Experimental Procedures. (B) Location of the cleavage sites found after V8 proteolysis of bisphosphorylated rat recombinant P2. The serine residues are indicated in boldface gray. The serines that were found by ES/MS coupled HPLC to be phosphorylated by GRK2 are underlined.

Table 2: Identification by ES/MS-Coupled HPLC of GRK2-Phosphorylated P2 Fragments[a]

peak (min)	ion number	measured mass (Da)	fragment	fragment mass (Da)	modifications	total mass (Da)
24.3	2	787.0 ± 0.1	58−64	786.9	none	786.9
26.1	2	1033.2 ± 0.2	105−112	1033.2	none	1033.2
27.4	2	958.1 ± 0.3	50−57	958.1	none	958.1
28.6	1	933.6	41−49	933.9	none	933.9
34.5	2	1664.2 ± 0.3	35−49	1684.8	none	1684.8
35.2	1	1874.4	41−57	1874.0	none	1874.0
35.9	2	4530.0 ± 0.7	65−113[1]	4530.0	none	4530.0
37.1	4	4015.5 ± 0.4	65−109	4515.5	none	4515.5
39.0	2	3514.6 ± 0.4	65−105[1]	3514.9	none	3514.9
	3	3386.0 ± 0.4	65−104	3385.8	none	3385.8
41.0	4	3697.3 ± 0.7	1−34	3697.1	none	3697.1
	4	3712.5 ± 0.4	1−34	3697.1	1 oxidation	3712.1
42.3	3	1877.7 ± 0.2	106−119[2]	1695.7	2 phosphates and 1 Na^+	1877.7
	1	1038.6	119−127[2]	1016.1	1 Na^+	1038.1
43.4	4	4408.0 ± 0.9	1−40	4408.0	none	4408.0
	4	4423.2 ± 0.7	1−40	4408.0	1 oxidation	4423.0
45.4	3	1283.2 ± 0.6	110−118	1080.1	2 phosphates and 2 Na^+	1284.1
	2	1563.6 ± 0.2	114−127[1]	1563.6	none	1563.6
	1	1217.6	117−127[1]	1218.3	none	1218.3
47.1	1	878.0	110−116	877.9	none	877.9
	1	785.8	121−127[1]	758.9	none	758.9
	2	2195.2 ± 1.1	110−127	2078.2	1 phosphate, 1 Na^+, and 1 oxidation	2195.2
47.8	2	1448.7 ± 0.6	116−127[1]	1347.4	1 phosphate and 1 Na^+	1449.4
50.1	4	5324.0 ± 0.5	1−49	5323.9	none	5323.9
	4	5339.1 ± 0.8	1−49	5323.9	1 oxidation	5338.9
53.7	4	4894.5 ± 1.0	1−45	4893.6	none	4893.6
	4	4908.2 ± 0.8	1−45	4893.6	1 oxidation	4909.5

[a] Composition of the peaks resulting from the separation by HPLC of V8 fragments of GRK2-biphosphorylated P2 (Figure 4A) was analyzed by ES/MS operated either in the negative ion mode or in the positive ion one [fragments found as ([1]) negative and positive ions; ([2]) negative ions only]. Masses of the fragments were calculated either from mono-charged ions or from several ions, and in this latter case, SEM is indicated. Attribution of the mass to a P2 fragment, of which the theoretical mass is shown, was performed using the ProMac software and considering the V8 protease specificity. The found cleavage sites are displayed in Figure 4B. Modifications were considered when no solution was obtained for another P2 fragment. That phosphorylation site(s) might exist outside of the C-terminal region has been systemically searched for but excluded. Total mass represents fragment- and modification-mass summation. The following masses were attributed to the corresponding modifications: +15 Da for an oxidation, +80 Da for a phosphate group, +22 Da for a Na^+.

the in vitro proteosynthetic activity of the ribosome (*12*). Here, we have shown that phosphorylation of P2 by GRK2 occurs on sites identical to those phosphorylated by CK2. Therefore, we could expect that phosphorylation of P2 by GRK2 would result in the same biological effect. Accordingly, we tested whether GRK2-phosphorylated P2 showed the same increase in proteosynthetic activity of the ribosome as after CK2 phosphorylation (*12*). Native P-proteins were

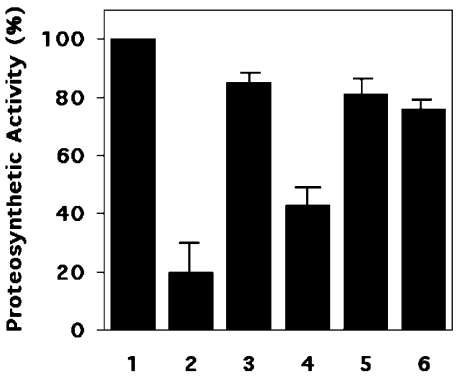

FIGURE 5: Effect of P2 phosphorylation on the proteosynthetic activity. 60S subunits were deprived selectively of native P-proteins by DMMA treatment (*14*), and the P2 phosphorylation effect was investigated after reconstitution of the DMMA core using combinations of unphosphorylated P1 and GRK2- or CK2-phosphorylated P2. Proteosynthetic activities were measured using the poly(U)-directed poly([^{14}C]-Phe) synthesis test as previously described (*12*). Under these conditions, translation depends only on the elongation step and the amount of 60S subunit is limiting. Bar 1 represents the proteosynthetic activity of 60S subunit treated the same way as core-particles but without DMMA. The following bars represent the proteosynthetic activity of the DMMA core deprived of native P1 and P2 (2), and the DMMA core after addition of (3) the native proteins (DMMA extract), (4) recombinant unphosphorylated P1 and P2, (5) recombinant unphosphorylated P1 and GRK2-phosphorylated P2, and (6) recombinant unphosphorylated P1 and CK2-phosphorylated P2. The translation rate observed with untreated 60S subunit was normalized to 100%. Results are the mean and SEM found in three different reconstitution experiments.

selectively extracted from 60S ribosomal subunits by a dimethylmaleic acid anhydride treatment (DMMA) to yield DMMA-core particles (*15*). Extraction of the native P-proteins was confirmed as previously described (*20*), and the obtained DMMA-core exhibited only 20% of the translational activity of the 60S subunit treated following the same protocol but without DMMA (Figure 5, compare bar 2 to bar 1). It is important to note that complete reconstitution of the translational activity is not possible, most likely due to DMMA also extracting additional proteins that contribute to the stabilization of the ribosome (*20*). However, these core particles keep the property of being reactivatable after reconstitution when the extracted proteins are added back (*20*). Addition of the DMMA-extract containing native P1 and P2 increased the translational activity to 85% (Figure 5, bar 3). Addition of the recombinant unphosphorylated P1 and P2 to the core-particle was not sufficient to restore significant activity (Figure 5, bar 4). However, when unphosphorylated recombinant P1 and, respectively, GRK2-phosphorylated recombinant P2 (bar 5) or CK2-phosphorylated recombinant P2 (bar 6) were added, translational activity was efficiently restored (Figure 5, compare bars 5 and 6 to bars 1 and 3). The rate of protein synthesis was essentially the same with both phosphorylated P1 and P2 [not shown; (*12*)] as it was with unphosphorylated P1 and phosphorylated P2, indicating that phosphorylation of P2 has a specific importance in the proteosynthetic process, whether it is catalyzed by CK2 or GRK2.

DISCUSSION

GRK2 phosphorylation of P2 following β_2AR stimulation provides evidence that suggests a signaling pathway leading to the phosphorylation of this protein in vivo in higher eukaryotes. The in vivo ability of GRK2 to phosphorylate P2 has been confirmed in vitro and is activated by the addition of G$\beta\gamma$ and PIP2 as is that of other known GRK2 substrates. It occurs on the sites found phosphorylated in vivo and phosphorylated in vitro by another kinase, CK2 (*11*). Moreover, phosphorylation of P2 by either GRK2 or CK2 results in the same increase of in vitro translational efficiency of the large ribosomal subunit.

The identification of the acidic proteins of the eukaryotic ribosome as phosphorylated proteins is not novel, hence the name P-proteins. However, the identity of the kinase(s) responsible for this phosphorylation in vivo has not been clearly established. Indeed, several kinases are able to phosphorylate these proteins. Most of the studies have been carried out in yeast, and different kinases, either cytoplasmic or bound to the ribosome (the ribosome-associated P-protein kinases), have been characterized (*21*, *22*) and listed in a recent review (*17*). For mammals, the ability of casein kinase 2 (CK2) to phosphorylate the P-proteins has been recognized for a long time (*10*) as well as the existence of endogenous activities in cellular extracts (see, for instance, *23*−*25*). A most interesting study, performed by Hasler and co-workers, showed that CK2 and ascitic cell extracts phosphorylate the P-proteins at the sites found phosphorylated in vivo (*11*). In addition, one study describes the phosphorylation of the P1 C-terminus as playing a functional role in yeast, targeting this protein for degradation (*26*). The originality of our finding is that a new kinase for the P-proteins is identified and a direct relation between an in vivo activation of this kinase and P2 phosphorylation is demonstrated.

The identification of P2 as an endogenous GRK2 substrate following isoproterenol stimulation raises the question of whether adrenergic receptors and GRKs regulate cellular protein synthesis. Effects of adrenergic stimulation on transcription have been well-documented and involve activation of CRE (cAMP responsive elements) and MAPKs (mitogen-activated protein kinases). However, studies relating translation and adrenergic stimulation are limited. Fuller and co-workers have demonstrated that stimulation of the α_1-adrenergic receptors in isolated cardiac myocytes from adult mice increases protein synthesis rates by 20−30% (*27*). Interestingly, this increase in protein synthesis is actinomycin D-insensitive, indicating that the effect occurs through translational and not transcriptional mechanisms. Likewise, adrenaline stimulated protein synthesis rates in anterogradely perfused hearts (*27*). Since α_1-adrenergic receptors are also able to interact with GRK2, we speculate that GRK2-mediated phosphorylation of P2 may provide a signal connecting extracellular hormonal activation and intracellular protein translation under these experimental conditions. Clinically known as cardiac hypertrophy, enlarged hearts are also seen following prolonged exposure to high levels of either α- or β-adrenergic catecholamines and are the result of increased protein synthesis (*28*). Moreover, pressure overload cardiac hypertrophy appears to result from stimulation of various Gq-coupled receptors. Therefore, understanding the relationships between adrenergic receptors, GRK2 activation, and ribosomal P-proteins could be important for elucidating the mechanisms relating catecholamine exposure, heart disease, and hypertension (*29*).

GRK2 is activated at the intracellular side of the plasmic membrane (*1*) whereas P2 plays its translational function in the ribosome. However, the existence of a cytoplasmic pool of P1 and P2 in rapid exchange with the ribosomal pool could provide an explanation for an involvement of the GRK2 pathway in regulating protein synthesis (*30*, *31*). The part played by P2 phosphorylation in the exchange rate has been postulated but is not clearly established (*12*, *17*, *32*, *33*). However, it cannot be excluded that GRK2 phosphorylation of P2 might be involved in an extraribosomal function of P2 as already shown for other ribosomal proteins (*34*, *35*). Moreover, as for eIF-2α, a protein involved in the initiation step of protein synthesis, P2 might participate in adrenergic-receptor signaling independent of its role in protein synthesis. Thus, eIF-2α was found associated with β_2AR at the plasma membrane in an agonist-dependent fashion (*36*). Overexpression of eIF-2α slightly enhances β_2AR activation of adenylyl cyclase, indicating that eIF-2α may function outside of its well-defined role in translation. The co-localization of these proteins at the plasma membrane implies that eIF-2α may play a role in receptor signaling in addition to its participation in the initiation of protein synthesis.

Another question is why P2 but not P1 was found phosphorylated in the cellular extract in a GRK2-dependent manner. Indeed, P1 is shown here to be a GRK2-phosphorylatable molecule and is likely to be associated with P2 whether it is free in the cytoplasm or bound to the ribosome by P0 (*37*). A simple explanation might be that P1 was phosphorylated but that this phosphorylation was not characterized. Another explanation could be that P1 is masked in these complexes whereas P2 is likely to be a protruding molecule (*37*).

In conclusion, we have demonstrated that P2 is phosphorylated in vivo in a GRK2- and agonist-dependent manner and that GRK2-phosphorylated P2 is able to stimulate the translational activity of purified ribosomes. Additional studies will be necessary to elucidate the pathway relating GPCR-induced GRK2 activation at the plasma membrane and P2 phosphorylation, on the one hand, and the actual involvement of GRK2-dependent phosphorylation of P2 in protein synthesis regulation, on the other.

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