Clathrin Box in G Protein-coupled Receptor Kinase 2*

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β1-Adrenergic receptor (β1-AR) shows the resistance to agonist-induced internalization. However, β1-AR can internalize as G protein-coupled receptor kinase 2 (GRK2) is fused to its carboxyl terminus. Internalization of the β1-AR and GRK2 fusion protein (β1-AR/GRK2) is dependent on dynamin but independent of β-arrestin and phosphorylation. The β1-AR/GRK2 fusion protein internalizes via clathrin-coated pits and is found to co-localize with the endosome that contains transferrin. The fusion proteins consisting of β1-AR and various portions of GRK2 reveal that the residues 498–502 in the carboxyl-terminal domain of GRK2 are critical to promote internalization of the fusion proteins. This domain contains a consensus sequence of a clathrin-binding motif defined as a clathrin box. In vitro binding assays show that the residues 498–502 of GRK2 bind the amino-terminal domain of clathrin heavy chain to almost the same extent as β-arrestin1. The mutation of the clathrin box in the carboxyl-terminal domain of GRK2 results in the loss of the ability to promote internalization of the fusion protein. GRK2 activity increases and then decreases as the concentration of clathrin heavy chain increases. Taken together, these results imply that GRK2 contains a functional clathrin box and directly interacts with clathrin to modulate its function.

Desensitization of G protein-coupled receptors (GPCRs) plays an important role in the physiological regulation of receptor signal transduction. β1-AR that belongs to a GPCR family is recognized as a model system to delineate the molecular mechanism of desensitization that consists of three processes: uncoupling, internalization (endocytosis), and down-regulation (1). Within seconds to minutes after agonist stimulation, β1-AR is phosphorylated by GRKs and cAMP-dependent protein kinase. Subsequent binding of β-arrestins to the GRK-phosphorylated β1-AR inhibits coupling of β1-AR with Gα. The agonist-bound β1-AR is also internalized from the plasma membrane to the intracellular endosome via clathrin-coated pits in a time period similar to that of uncoupling. Internalization is the process that contributes to functional resensitization by promoting dephosphorylation and recycling of the receptor to the plasma membrane (2, 3). However, it has been reported that internalization contributes not only to uncoupling and recycling of receptors but also to down-regulation by promoting degradation of the receptors in lysosomes (4–7).

Several studies have reported that GRKs and β-arrestins are implicated in internalization of many GPCRs. Overexpression of GRK2 enhanced internalization of m2AChR, whereas overexpression of a dominant-negative GRK2 inhibited it (8). Similarly, overexpression of GRK2 rescued agonist-promoted internalization of the internalization-defective β2-AR mutant (Y326A β2-AR), whereas the β2-AR mutant lacking putative GRK2 phosphorylation sites was not internalized (9). Overexpression of β-arrestin1 or β-arrestin2 rescued internalization of Y326A β2-AR, whereas dominant-negative β-arrestins inhibited internalization of WT-β2-AR (10). These results show that GRK and β-arrestin play an essential role in internalization of some GPCRs. It has been reported that β-arrestins promote internalization by interacting not only with GPCRs but also with clathrin, the major component of the clathrin-based endocytic machinery (11). β-Arrestins bind clathrin by interaction of a consensus clathrin-binding motif in the carboxyl-terminal domain with the amino-terminal domain of the clathrin heavy chain (12).

The formation of vesicles for transport is mediated by coat proteins associated with integral membrane proteins (13). The clathrin-coated vesicle is the most characterized example, which consists mainly of clathrin and protein complexes termed adaptor protein-2 (AP-2) that forms at the plasma membrane when membrane receptors internalize (14). The regulatory proteins such as β-arrestins and amphiphysins, which have been demonstrated to interact with clathrin, have conserved sequences within the clathrin-binding regions (12, 14). Alignment of these sequences defines a consensus motif for clathrin binding that consists of acidic and bulky hydrophobic residues and conforms to the canonical sequence L(L/I)D/E/N(L/I)F(X)D/E. This region can bind to the amino-terminal domain of clathrin heavy chain (16).

In contrast with β1-AR, β1-AR undergoes only slight internalization upon agonist stimulation (17). A lower affinity of β-arrestins for β1-AR than for β2-AR can explain the differential behavior of internalization between β1-AR and β2-AR. We determined the interaction of β-arrestins with β1-AR by several methods, and we found that β1-AR has a lower affinity for β-arrestins than β2-AR (18). In the course of analysis of the resistance of β1-AR to agonist-induced internalization, we have found that the β1-AR/GRK2 fusion protein internalized upon...
agonist stimulation, which was independent of β-arrestins but dependent on dynamin. This unique internalization behavior of the fusion protein turned out to be due to the existence of clathrin box in GRK2.

In the present study, we demonstrate that the carboxyl-terminal domain of GRK2 contains a consensus motif of clathrin binding shared with β-arrestins, amphipapsins, and AP-2. GRK2 directly interacts with clathrin via this clathrin-binding motif, and the phosphorylating ability of GRK2 is regulated by clathrin binding. It suggests that GRK2 participates in agonist-promoted internalization in more direct ways.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco's modified Eagle's medium (DMEM), phosphate-free DMEM, and LipofectAMINE were obtained from Life Technologies, Inc. Fetal bovine serum was purchased from JRH Biosciences (Lenexa, KS). Plasmids encoding for human β1-AR and β2-AR, rat GRK2, rat β-arrestin1, and rat β-arrestin2 were kindly provided by Dr. R. J. Lefkowitz (Duke University). WT-dynamin and dominant-negative dynamin (dynamin K44A) were kindly provided by Dr. S. L. Schmid (Scripps Research Institute). HEK293 cell and restriction enzymes were from Takara Shuzo (Kyoto, Japan). Propranolol, (−)-isoproterenol hydrochloride, bovine albumin, chloroquine diphosphate salt, and isopropyl-1-thio-β-D-galactopyranoside were obtained from Sigma. [3H]Methionine, HyperfilmTM, and glutathione-Sepharose beads were from Amersham Pharmacia Biotech. Leptpin was purchased from Peptide Institute Inc. (Osaka, Japan). [3H]CGP-12177 and [32P]PO4 were from PerkinElmer Life Sciences. TNT coupled transcription/translation system was obtained from Promega (Madison, WI). Anti-HA antibody was purchased from Roche Molecular Biochemicals. Alexa FluorTM 594-conjugated human transferrin was purchased from Molecular Probes.

**Plasmid Construction**—The constructs of the HA-tagged β1- and β2-ARs were described by Sato et al. (19). The β1- and β2-AR/GRK2 fusion proteins were constructed as follows. First, various coding regions of proteins were constructed as follows. First, various coding regions of GRK2 were amplified and ligated into EcoRI/HindIII sites of pBluescript SKII (−). The PCR product was subcloned into pGEX-4T1. The resulting fusion protein was expressed and purified by the same way as GST-GRK2CT fusion proteins. Phosphorylation of β-AR/GRK2 fusion protein was performed with a slight modification of Kurose and Leffkowitz (21). HEK293 cells transiently expressing βARs were labeled with 100 μCi/ml [32P]PO4 for 1 h in serum- and phosphate-free DMEM. The cells were then stimulated by 10 μM isoproterenol for 10 min, and after extensive washing cells were scraped into buffer (10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate) containing 10 μg/ml leupeptin and 10 μg/ml soybean trypsin inhibitor. The βARs were solubilized by stirring for 1 h at 4°C and were centrifuged at 15,000 rpm for 30 min at 4°C. The solubilized βARs were incubated with 2 μg of anti-HA antibody and immunoprecipitated by incubating with protein A-Sepharose for 1 h. The immunoprecipitated receptors were separated from protein A-Sepharose by incubating them at 37°C for 1 h and resolved by SDS-PAGE. The phosphorylated receptors were detected with Fuji BAS 1800.

**Confocal Microscopy**—Confocal microscopy was performed as described (18). HEK293 cells were transfection with the epitope-tagged and GRK2-fused β1-AR. Cells expressing the β1-AR/GRK2 fusion protein were plated onto 35-mm glass bottom dishes. On the day of experiment, the medium was replaced with serum-free DMEM containing 20 mM HEPES, pH 7.4. The cells were then incubated for 30 min with 40 μg/ml Isoflurane, and Alexa FluorTM 594-conjugated transferrin. After transferrin was endocytosed, cells were stimulated by 10 μM isoproterenol for 30 min. Cells were washed, fixed with 4% formaldehyde, and permeabilized in 0.1% Triton X-100, and HA epitope at the amino terminus of the β1-AR/GRK2 fusion protein was detected by rat anti-HA-antibody and fluorescein isothiocyanate-conjugated anti-rat antibody. Confoical images were obtained with two excitation wavelengths, 485 nm for β1-AR/GRK2 and 515 nm for transferrin. Signals were collected with a Laser Scanning Confocal Imaging System from Bio-Rad using Nikon CFI PLN APOC- HROMAT 60×/w. The β1-AR/GRK2 was localized on the plasma membrane before agonist stimulation (data not shown).

**Binding of In Vitro Translated Clathrin to GST Fusion Proteins**—A cDNA fragment encoding residues 1–580 of human clathrin heavy chain was cloned by reverse transcriptase-polymerase chain reaction using the reverse transcription/translation system and Pfu DNA polymerase according to the manufacturer's instructions. The PCR product was subcloned into BamHI and XhoI sites of the pBluescript SKII (−) vector and sequenced. The resulting amino-terminal domain of clathrin heavy chain was translated and translated in vitro using TNT quick-coupled transcription/translation system in the presence of [35S]methionine according to the manufacturer's instructions. The GST fusion proteins were immobilized on glutathione-Sepharose beads by incubating for 60 min at 4°C and then incubated for 60 min with in vitro translated clathrin in the binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM KCl, 2 mM MgCl2, 0.1 mM CaCl2, 10% glycerol, 0.1% bovine serum albumin, 1% Triton X-100) at 4°C. After washing five times with the binding buffer, bound proteins were eluted and resolved by SDS-PAGE. The radiolabeled clathrin was detected by autoradiography and quantified by Fuji BAS 1800.

**Purification of GRK2 and Phosphorylation of m2AChR**—Purification of GRK2 and phosphorylation of m2AChR by GRK2 and m2AChR were expressed in Sf9 cells with the baculovirus expression system (18). The expression levels of βARs in the present study were 1080 ± 103 fmol/mg (WT-β2-AR), 742 ± 93 fmol/mg (β2-AR/GRK2), 694 ± 51 fmol/mg (WT-β1-AR), and 497 ± 83 fmol/mg (β1-AR/GRK2).

**Phosphorylation of β1-AR/GRK2 fusion protein**—The β1-AR/GRK2 fusion protein was phosphorylated by a slight modification of Kurose and Leffkowitz (21). HEK293 cells transiently expressing β1-AR were labeled with 100 μCi/ml [32P]PO4 for 1 h in serum- and phosphate-free DMEM. The cells were then stimulated by 10 μM isoproterenol for 10 min, and after extensive washing cells were scraped into buffer (10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate) containing 10 μg/ml leupeptin and 10 μg/ml soybean trypsin inhibitor. The βARs were solubilized by stirring for 1 h at 4°C and were centrifuged at 15,000 rpm for 30 min at 4°C. The solubilized βARs were incubated with 2 μg of anti-HA antibody and immunoprecipitated by incubating with protein A-Sepharose for 1 h. The immunoprecipitated receptors were separated from protein A-Sepharose by incubating them at 37°C for 1 h and resolved by SDS-PAGE. The phosphorylated receptors were detected with Fuji BAS 1800.
subjected to 10% SDS-PAGE, and the radioactive m2AChR was detected and quantified by Fuji BAS 1800.

RESULTS

Agonist-induced Internalization of βAR/GRK2 Fusion Proteins—We have determined agonist-induced internalization by measuring the numbers of WT-βARs or the βAR/GRK2 fusion proteins remaining at the cell surface. Consistent with the previous report (17, 18, 23), the number of β1ARs was not decreased although the number of β2ARs was decreased about 30% by isoproterenol stimulation for 30 min (Fig. 1). In contrast with WT-βAR, the β1AR/GRK2 fusion protein that GRK2 was fused to at the carboxyl terminus of β1AR was internalized by isoproterenol stimulation (Fig. 1A). The β2AR/GRK2 fusion protein internalized to a much greater extent than WT-β2AR (Fig. 1B). As receptor phosphorylation plays a central role in agonist-induced internalization (9), we determined the phosphorylation states of β1AR, β2AR, and β1AR/GRK2 fusion protein. When β1AR was expressed in HEK293 cells, β2AR showed the increased phosphorylation by agonist stimulation (Fig. 2A). Under the same conditions, β2AR was also phosphorylated by agonist stimulation, although the degree of phosphorylation was much smaller than that of β1AR. In contrast to β1AR, β2AR/GRK2 fusion protein was efficiently phosphorylated by agonist stimulation (Fig. 2B). This result suggests that β2AR has a lower affinity for GRK2 than β1AR. It also raises the possibility that the enhanced internalization of the β1AR/GRK2 fusion protein is caused in part by the increased phosphorylation of β1AR.

As the increased phosphorylation of the receptor recruits β-arrestins to promote internalization of the receptor, we determined whether the β1AR/GRK2 fusion protein was internalized via β-arrestin- and dynamin-dependent pathways. The carboxy-terminal domains of β-arrestins interact with clathrin and AP-2 and promote internalization of the agonist-bound and -phosphorylated receptors (24). Therefore, the carboxy-terminal domains of β-arrestins (for instance β-arrestin2-(284–409)) can behave as dominant-negative mutants. Dynamin is a GTPase that pinches off the vesicles at the necks of clathrin-coated pits and is therefore essential for the formation of clathrin-coated vesicles (25, 26). Therefore, dynamin mutagenesis at Lys44 that is important for GTP binding (dynamin K44A) works as a dominant-negative mutant. As shown in Fig. 3A, overexpression of β-arrestin2-(284–409) and dynamin K44A significantly attenuated agonist-promoted internalization of β1AR. In consistent with β2AR, dynamin K44A completely prevented agonist-promoted internalization of the β1AR/GRK2 fusion protein (Fig. 3B). However, β-arrestin2-(284–409) was without effect on internalization of the β2AR/GRK2 fusion protein. Consistent with the inability of β-arrestin2-(284–409) to inhibit internalization, stimulation of the β2AR/GRK2 fusion protein did not translocate β-arrestin2-enhanced green fluorescent protein to the plasma membrane (data not shown). Internalization of the β1AR/GRK2 fusion protein was not inhibited by a dominant negative β-arrestin2 mutant (β-arrestin2-(284–409)), and stimulation of the β2AR/GRK2 fusion protein did not translocate β-arrestin2-enhanced green fluorescent protein to the plasma membrane. Therefore, it is concluded that the enhanced phosphorylation of the β1AR/GRK2 fusion protein (shown in Fig. 2) does not result in the increased binding of β-arrestins. These results also suggested that the β1AR/GRK2 fusion protein internalizes via a dynamin-dependent but β-arrestin-independent pathway. Next we examined the contribution of GRK2 fusion gene to internalization behavior of β1AR that efficiently internalizes upon agonist stimulation. The effect of GRK2-induced phosphorylation and β-arrestin binding on internalization was blocked by the expression of β-arrestin2-(284–409). Therefore, we can determine the contribution of GRK2 fusion gene to internalization. β-arrestin2-(284–409) almost completely blocked internalized binding of β1AR. However, β-arrestin2-(284–409) partially inhibited internalization of the β2AR/GRK2 fusion protein (Fig. 3C). This result suggests that GRK2 induces internalization by itself in a phosphorylation-independent manner when GRK2 is fused to the receptors. This result also indicates that the β2AR/GRK2 fusion protein internalizes via at least two pathways. To examine whether internalization of the fusion protein is dependent on clathrin, internalization via clathrin-coated pits was blocked by various treatments (27, 28). Stimulation with isoproterenol for 30 min promoted internalization of the β2AR/GRK2 fusion protein by ~30% (Fig. 4). However, the incubation with 0.5 mg/ml concanavalin A, or acetic acid resulted in the blockade of internalization of the β1AR/GRK2 fusion protein. Together, these results indicate that β1AR can be internalized when GRK2 was fused to the carboxyl terminus of β1AR and that internalization of the fusion protein is dependent on clathrin and dynamin but independent of β-arrestin.

Visualization of Redistribution of Isoproterenol-stimulated βAR/GRK2 Fusion Protein—To determine the subcellular localization of the β1AR/GRK2 fusion protein after agonist stimulation, we performed confocal microscopy to compare the localization of the fusion protein with that of transferrin, a well established marker of early and recycling endosomes (29, 30). In the resting state, the fusion protein was localized at the plasma membrane and showed the different distributions from transferrin that was internalized and accumulated in early
endosomes (data not shown). After agonist stimulation, the fusion protein appeared in endocytic vesicles that were randomly distributed throughout cytosol and co-localized with transferrin (Fig. 5, co-localization is shown by the appearance of the yellow color when the green color is coincident with the red color). This observation supports the idea that the βAR/GRK2 fusion protein is really internalized by agonist stimulation and sorted to endocytic vesicles containing transferrin.

**Internalization Behavior of Various Mutants of βAR/GRK2 Fusion Protein**—To determine which region of GRK2 is responsible for differential behavior of agonist-promoted internalization of the fusion protein, we constructed various βAR/GRK2 mutants and determined the internalization behavior of them. The structures of βARs were 59 ± 12 fmol/mg (none), 370 ± 120 fmol/mg (βAR), 340 ± 100 fmol/mg (β2AR), and 340 ± 59 fmol/mg (β2AR/GRK2) of three experiments in duplicate. A, β2AR but not β1AR was strongly phosphorylated by isoproterenol (Iso) stimulation. B, the β2AR/GRK2 fusion protein was also phosphorylated by isoproterenol stimulation, and no phosphorylation was observed in control HEK293 cells.

**Fig. 2.** Phosphorylation of β2AR, β1AR and β1AR/GRK2 fusion protein. HEK293 cells transiently expressing β2AR and β1AR (A) or β2AR and β1AR/GRK2 fusion protein (B) were labeled with [32P]PO4 and stimulated by 10 μM isoproterenol. The receptors were solubilized, immunoprecipitated, and subjected to SDS-PAGE. The experiments were repeated three times, and similar results were obtained. The expression levels of βARs were 59 ± 12 fmol/mg (none), 370 ± 120 fmol/mg (βAR), 340 ± 100 fmol/mg (β2AR), and 340 ± 59 fmol/mg (β2AR/GRK2) of three experiments in duplicate. A, β2AR but not β1AR was strongly phosphorylated by isoproterenol (Iso) stimulation. B, the β2AR/GRK2 fusion protein was also phosphorylated by isoproterenol stimulation, and no phosphorylation was observed in control HEK293 cells.

**Fig. 3.** Effect of the dominant-negative mutants of β-arrestin2 and dynamin on agonist-promoted internalization of βAR, β2AR/GRK2, or β1AR/GRK2 fusion proteins in HEK293 cells. HEK293 cells were co-transfected with a plasmid coding for either WT-β2AR (A) or β1AR/GRK2 fusion protein (B) or β1AR/GRK2 fusion protein (C) and a plasmid encoding β-arrestin2-(284–409) (β-arrest2CT) or dynamin K44A (DynK44A). Cells were stimulated with 10 μM isoproterenol for 30 min at 37 °C, and the receptors remaining on the cell surface were determined by [3H]CGP-12177 binding assay. The data are represented as described in the legend of Fig. 1.
The carboxyl-terminal domain of GRK2 is necessary to agonist-promoted internalization of the β1AR. This result suggests that the carboxyl-terminal domain of GRK2 is necessary to promote internalization of the β1AR/GRK2 fusion protein. After searching a motif for internalization in the carboxyl-terminal domain of GRK2, we found a potential clathrin-binding motif LLDSDF at positions 498–502. We suspected that direct interaction of the carboxyl-terminal domain of GRK2 with clathrin promotes internalization of β1AR. Therefore, we mutated the amino acids in this region to alanine residues (Fig. 6A). When 496LLDSDF in the carboxyl-terminal domain of GRK2 was mutated to alanine and fused to the β1AR, this mutant did not internalize upon agonist stimulation (Fig. 6B). This result raised the possibility that 496LLDSDF in GRK2 played an essential role in agonist-promoted internalization of the β1AR/GRK2 fusion proteins by direct interaction with clathrin.

**Interaction of In Vitro Translated Clathrin with Carboxyl Terminus of GRK2**—The results in the previous sections indicated that GRK2 directly interacts with clathrin via its clathrin-binding motif in the carboxyl-terminal domain. It has been reported that the clathrin-binding motif binds to the amino-terminal domain of clathrin heavy chain. Therefore, we determined the ability of the carboxyl-terminal domain of GRK2 to bind clathrin. The GST fusion proteins of β-arrestin1 or the carboxyl-terminal domain of GRK2 were individually immobilized on glutathione-Sepharose beads and incubated with 58S-labeled clathrin generated by *in vitro* translation. The bottom panel of Fig. 7A shows the Coomassie gel staining of GST fusion proteins that were used for the clathrin binding experiment. Although β-arrestin1-GST, GRK2CT-GST, and GRK2CT(AAAAAA)-GST fusion proteins showed some degradation or truncation products, major bands of these fusion proteins were products of full-length inserts based on molecular weights of these products. Clathrin did not bind to GST alone (upper panel of Fig. 7A). In contrast with GST alone, clathrin bound strongly to β-arrestin1, consistent with the previous report (16). Clathrin also strongly bound to the carboxyl-terminal domain of GRK2. When the amino acids of clathrin-binding motif in the carboxyl-terminal domain of GRK2 were mutated to alanine (GRK2CT-498LLDSDF → GRK2CT-498AAAAA), the clathrin binding to the carboxyl-terminal domain was greatly decreased (Fig. 7B). Considering the integrity and amounts of three fusion proteins (Fig. 7A), it is concluded that GRK2CT binds clathrin almost same degree as β-arrestin1, and alanine-substituted GRK2CT mutant binds much less than the other two fusion proteins. These results clearly demonstrate that GRK2 has a clathrin-binding motif in its carboxyl-terminal domain and interacts with clathrin heavy chain through that motif.

**Regulation of GRK2-mediated m2AChR Phosphorylation by Clathrin**—It has been reported very recently that class II PI3K-C2α is activated by clathrin (31). Therefore, we determined whether interaction of GRK2 with clathrin resulted in the enhanced phosphorylation activity of GRK2 toward agonist-bound receptor. As GRK2 can phosphorylate not only β2AR but also m2AChR in *in vitro* and *in vivo*, m2mAChR was used for *in vitro* phosphorylation experiments by GRK2. The fusion protein of clathrin heavy chain residues 1–580 with GST was expressed in *Escherichia coli* and purified from it. The purified preparation was not a full-length product (Fig. 8A). Because it has been reported that the amino-terminal portion is essential for interaction with clathrin-binding proteins (31), we used this fusion protein for the functional experiment. GRK2 phosphorylated agonist-bound m2AChR to some extent in the absence of any activator (Fig. 8B). The phosphorylation activity of GRK2 increased and then decreased as the concentration of the clathrin-GST fusion protein increased. This result indicates that clathrin modulates the function of GRK2.

**DISCUSSION**

In the present study, we demonstrated that GRK2 contains a functional clathrin binding domain. Our results provide GRK2 with a new function other than phosphorylation activity toward activated GPCRs. Many GPCRs are phosphorylated by GRKs in an agonist-dependent manner as a major mechanism of receptor regulation. There are six members of the GRK family (32). GRKs belong to the serine/threonine kinase family and show the property to phosphorylate agonist-occupied receptors, thereby triggering desensitization. GRK2 is the primary GRK expressed in the heart and is studied most extensively to date. Phosphorylation of GPCRs by GRK2 and subsequent binding of β-arrestins is one of the mechanisms leading to uncoupling from G proteins. The binding of β-arrestins to almost all GPCRs appears to enhance internalization through interaction of β-arrestins with clathrin and AP-2 (11, 24).
The vesicles for internalization consist of several proteins, which formed at the plasma membrane. The most extensively characterized proteins in the vesicles are clathrin and the protein complex termed AP-2 (33, 34). Clathrin is the complex of three heavy chains and three light chains that polymerizes to form the cargo for internalization. AP-2 recruits the internalizing membrane proteins to the coated pits and also mediates attachment of clathrin to the membrane. β-Arrestin (12, 16) as well as other proteins mentioned in Fig. 9 have been demonstrated to interact with clathrin. These clathrin-interacting proteins have conserved amino acid sequences for clathrin binding. This clathrin-binding motif is defined as a clathrin box that conforms to the canonical sequence L(L/I)(D/E/N)(L/F)(D/E) (Fig. 9). In the present study, we found that GRK2 also has the sequence 498LLDSD502 similar to the canonical sequence of clathrin box in its carboxyl-terminal domain. As AP-2 and β-arrestins bind to the amino-terminal domain of clathrin heavy chain (17, 24), we determined whether GRK2 also binds to the amino-terminal domain of clathrin heavy chain. We found that the carboxyl-terminal domain of GRK2 bound clathrin essentially the same extent to that of β-arrestin1. These results suggest that clathrin box of GRK2 is functional not only in binding of clathrin but also in enhancement of agonist-promoted internalization.

GRK2 is divided into three domains as follows: amino-terminal domain, catalytic domain, and carboxyl-terminal regulatory domain. The amino-terminal domain contains regulators of G protein signaling (RGS) domains specific for Gαq, and the carboxyl-terminal domain contains Gβγ binding domain and pleckstrin homology (PH) domain that binds phosphatidylinositol 4,5-diphosphate (35, 36). The present study adds another functional domain to GRK2, which interacts with clathrin and helps agonist-promoted internalization.

GPCRs internalize via several pathways. The most characterized and established internalization is the β-arrestin- and dynamin--dependent pathway such as β2AR internalizes. GRK-mediated phosphorylation triggers internalization by promoting the binding of β-arrestins to the phosphorylated receptors and subsequent interaction with clathrin and AP-2. On the contrary, some GPCRs such as angiotensin II receptor and m2AChR internalize via β-arrestin-- and dynamin-independent pathway. In contrast to these GPCRs, m1, m3, and m4AChRs internalize via β-arrestin-independent but dynamin-dependent pathway (37, 38). Direct interaction of GRK2 with clathrin may be involved in internalization of these mAChRs via dynamin-dependent pathway.

Affinity of substance P receptor for GRK2 seems to be higher than that for other GPCRs (39), because activation of substance P receptor but not other types of GPCRs including β2AR and angiotensin II receptor induces translocation of GRK2 to the plasma membrane. A plausible explanation for this intriguing observation is that substance P receptor has higher affinity for

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**Fig. 6. Structures and internalization behavior of various β2AR/GRK2 fusion proteins.** A, schematic representation of WT-GRK2 and various portions of GRK2 expressing as the fusion proteins with β2AR. B, internalization behavior of various β2AR/GRK2 fusion proteins. The fusion proteins were transiently transfected in HEK293 cells. After cells were stimulated with 10 μM isoproterenol for 30 min, the binding activities were determined by incubating with [3H]CGP-12177. The data are represented as described in the legend of Fig. 1. The data are mean ± S.E. of five independent experiments carried out in duplicate. NT, amino terminus; CT, carboxyl terminus.

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GRK2 than other GPCRs (39). Therefore, GRK2 translocating to the plasma membrane may directly involve internalization of substance P receptor by interacting with clathrin.

Gaidarov et al. (31) reported very recently that clathrin activates the class II PI3K-C2α and regulates membrane trafficking via PI3K-C2α. The class I PI3K is involved in postendocytic trafficking of the receptors and is sensitive to wortmannin and LY294002. PI3K-C2α belongs to class II PI3K and shows the resistance to PI3K inhibitors. Because PI3K-C2α is activated by clathrin and the consequent production of PIP3 can recruit various PIP3-binding proteins, it was proposed that clathrin helps to stimulate endocytosis by the increased formation of PIP3 (31). In the present study, we demonstrated that the amino-terminal domain of clathrin heavy chain binds the carboxyl-terminal region of GRK2 and modulates GRK2 phosphorylation activity. GRK2 activity was regulated by clathrin in a bell-shaped manner. Low concentration of clathrin-GST fusion protein increased, and high concentration decreased GRK2 phosphorylation activity.

**Fig. 7. Interaction of carboxyl-terminal domain of GRK2 with clathrin.**

A, Coomassie staining gel of the fusion proteins bearing portions of GRK2, $^{[35}S\text{]methionine-labeled}$ amino-terminal domain of clathrin to these GST fusion proteins. Each of the fusion protein (10 μg) was subjected to SDS-PAGE and stained with Coomassie Blue. Each of the fusion proteins (100 μg) was immobilized on glutathione-Sepharose beads, and the binding activities were determined. B, quantification of clathrin bound to the fusion proteins by image analyzer. The data are represented as a percentage of binding by dividing the amounts of clathrin bound to the fusion proteins by the total amount of clathrin adding to the columns. The data represent the mean ± S.E. of five separate experiments.

**Fig. 8. The regulation of GRK2 phosphorylating activity by clathrin.**

A, Coomassie gel staining of clathrin-GST or GST. Five μg of clathrin-GST or GST was subjected to 12% SDS-PAGE and stained by Coomassie Blue. B, purified and reconstituted m2AChR was phosphorylated by GRK2 in the presence of the indicated concentration of clathrin-(1–580)-GST fusion protein or GST alone. Phosphorylation of m2AChR by GRK2 was quantified by Fuji BAS 1800 and represented as fold stimulation of basal phosphorylation activity of GRK2. Data are represented as mean ± S.E. of three to five experiments.
but also is likely to function as a clathrin adaptor, just like only phosphorylates the receptor in an active conformation for resolving these issues. Our findings imply that GRK2 not phosphorylation of tubulin and leads to cytoskeletal rearrange-
ment (15). Therefore, it is interesting to speculate that phosphorylates a cytoskeletal protein, tubulin, and modulates its function (15). Thereafter, it is interesting to speculate that activation of GRK2 by clathrin results in the enhanced phosphorylation of tubulin and leads to cytoskeletal rearrangement for internalization. Further studies will be necessary for resolving these issues. Our findings imply that GRK2 not only phosphorylates the receptor in an active conformation but also is likely to function as a clathrin adaptor, just like β-arrestins and AP-2. GRK2 may be more directly involved in the sorting events than previously envisioned. In conclusion, we demonstrated that GRK2 directly interacts with clathrin via the clathrin box located at the carboxy-terminal domain of GRK2. The clathrin box is a consensus motif shared with AP-2, β-arrestins, and amphiphysins to interact with the amino-terminal domain of clathrin heavy chain. Conservation of this motif is likely to account for the ability of all these proteins to bind clathrin despite their structural diversity. Therefore, β1AR can internalize upon agonist stimulation when GRK2 was fused to the carboxyl terminus of β1AR.

### Clathrin-Box

| Consensus | L (L1) | (D,E,N) | (L,F) | (D,E) |
|-----------|--------|---------|-------|-------|
| β-arrestin 1 | 373 | VDTN | LIELD | TNDD385 |
| β-arrestin 2 | 369 | VDTN | LIEFE | TNYA381 |
| AP-1 β1 | 628 | LLLG | DLLNL | DGP640 |
| AP-2 β1 | 627 | LLLG | DLLNL | DGP639 |
| AP-3 β3A | 814 | KDVS | LLDLD | DFP682 |
| AP-3 β3B | 803 | KESI | LLDLD | DFTP815 |
| amphiphysin I | 347 | KEET | LLDLD | FDPE359 |
| amphiphysin II | 386 | EQAS | LLDLD | FDPL398 |
| epsin 1 | 476 | PNAALV | DLDLS | 8488 |

**GRK2**

494 KGK LLLDSQEL Y506

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### REFERENCES

1. Böhm, S. K., Grady, E. F., and Bunnett, N. W. (1997) *Biochem. J.* 322, 1–18
2. Yu, S. S., Lefkowitz, R. J., and Hausdorff, W. P. (1993) *J. Biol. Chem.* 268, 337–341
3. Lefkowitz, R. J., Pitcher, J., Krueger, K., and Daaka, Y. (1998) *Adv. Pharma-
col.* 41, 416–420
4. Kallal, L., Gagnon, A. W., Penn, R. B., and Benovic, J. L. (1998) *J. Biol. Chem.* 273, 322–329
5. Law, P. Y., Hom, D. S., and Loh, H. H. (1994) *J. Biol. Chem.* 269, 4096–4104
6. Hoxie, J. A., Abuja, M., Belmonte, E., Pizarro, S., Parton, R., and Brass, L. F. (1996) *J. Biol. Chem.* 271, 15011–15016
7. Ferguson, S. S. G., Downey, W. E., III, Colapietro, A.-M., Barak, L. S., Ménard, L., and Caron, M. G. (1996) *Science* 271, 363–366
8. Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) *Nature* 383, 447–455
9. Krupnick, J. G., Goodman, O. B., Jr., Keen, J. H., and Benovic, J. L. (1997) *J. Biol. Chem.* 272, 15011–15016
10. Rothman, J. E., and Wieland, F. T. (1996) *Science* 272, 227–234
11. Dell’Angelica, E. C., Klumperman, J., Stoorvogel, W., and Bonifacino, J. S. (1998) *Science* 280, 431–434
12. Carman, C. V., Som, T., Kim, C. M., and Benovic, J. L. (1998) *J. Biol. Chem.* 273, 20308–20316
13. Goodman, O. B., Jr., Krupnick, J. G., Gurevich, V. V., Benovic, J. L., and Keen, J. H. (1997) *J. Biol. Chem.* 272, 15017–15022
14. Suzuki, T., Nguyen, C. T., Nambel, F., Bonin, H., Valiquette, M., Frielle, T., and Bovier, M. (1992) *Mol. Pharmacol.* 41, 542–548
15. Shiina, T., Kawasaki, A., Nagao, T., and Kurose, H. (2000) *J. Biol. Chem.* 275, 66662–66667
16. Sato, Y., Kurose, H., Isogaya, M., and Nagao, T. (1996) *Eur. J. Pharmacol.* 315, 363–367
17. Furukawa, H., and Haga, T. (2000) *J. Biochem. (Tokyo)* 127, 151–161
18. Igurose, H., and Lefkowitz, R. J. (1994) *J. Biol. Chem.* 269, 10993–10999
19. Haga, K., Tsuga, H., and Haga, T. (1997) *Biochemistry* 36, 1315–1321
20. Green, S. A., and Liggitt, S. B. (1994) *J. Biol. Chem.* 269, 26215–26219
21. Laporte, S. A., Oakley, R. H., Zhang, J., Holt, J. A., Ferguson, S. S. G., Caron, M. G., and Barak, L. S. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 3712–3717
22. Damke, H. (1996) *FEBS Lett.* 389, 48–51
23. Urrutia, R., Henley, J. R., Cook, T., and McNiven, M. A. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 377–384
24. Hansen, S. H., Sandvig, K., and van Deurs, B. (1993) *J. Cell Biol.* 121, 61–72
25. Pippig, S., Andexinger, S., and Lohse, M. J. (1995) *Mol. Pharmacol.* 47, 653–676
26. Dunn, K. W., McGraw, T. E., and Maxfield, F. R. (1989) *J. Cell Biol.* 109, 3303–3314
27. Hopkinis, C. R., and Troubbridge, I. S. (1983) *J. Cell Biol.* 97, 508–521
28. Gaidarov, I., Smith, E. M. K., Dominn, J., and Keen, J. H. (2001) *Mol. Cell* 7, 433–449
29. Pitcher, J. A., Freedman, N. J., and Lefkowitz, R. J. (1998) *Annu. Rev. Bio-
chem.* 67, 653–692
30. Schmid, S. L. (1997) *Annu. Rev. Biochem.* 66, 511–548
31. Brodsky, F. M. (1997) *Trends Cell Biol.* 7, 175–179
32. Carman, C. V., Parent, J.-L., Day, P. W., Pronin, A. N., Sternweis, P. M., Wedegaertner, P. B., Gilman, A. G., Benovic, J. L., and Kozasa, T. (1999) *J. Biol. Chem.* 274, 34483–34492
33. Touhara, K., Koch, W. J., Hawes, B. E., and Lefkowitz, R. J. (1995) *J. Biol. Chem.* 270, 17000–17005
34. Lee, K. B., Pals-Rylaarsdam, R., Benovic, J. L., and Hoyer, M. M. (1998) *J. Biol. Chem.* 273, 12967–12972
35. Vogler, O., Boguth, K., Wolske, C., Krummenerl, P., Jakobs, K. H., and van Koppen, C. J. (1998) *J. Biol. Chem.* 273, 12155–12160
36. Barak, L. S., Karbasi, K., Feng, X., Caron, M. G., and Kwartha, M. M. (1999) *J. Biol. Chem.* 274, 7565–7569
