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Overexpression of Wild-Type and Catalytically Inactive Forms of GRK2 and GRK6 Fails to Alter the Agonist-Induced Phosphorylation of the C5a Receptor (CD88): Evidence That GRK6 Is Autophosphorylated in COS-7 Cells

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The G protein-coupled receptor kinase family comprises six members (GRK1 to GRK6) that phosphorylate and desensitize a number of agonist-occupied G protein-coupled receptors. Overexpression of the dominant negative mutant GRK2-K220R is often accompanied by an inhibition of the agonist-mediated phosphorylation of G protein-coupled receptors. In the case of the C5a receptor (C5aR), the overexpression of wild-type GRK2 or GRK6 as well as of catalytically inactive forms of these kinases (GRK2-K220R and GRK6-K215R) failed to increase or to inhibit the agonist-mediated phosphorylation of C5aR, respectively. Replacement of Lys^{215} by an arginine residue in GRK6 yielded a protein with a relative molecular mass of 63 kDa, whereas wild-type GRK6 had a relative molecular mass of 66 kDa on polyacrylamide gel. The mutations S484D and T485D in the catalytically inactive mutant GRK6-K215R resulted in a protein (GRK6-RDD) with the same electrophoretic mobility as wild-type GRK6. Furthermore, in the absence of phosphatase inhibitors, GRK6 was rapidly converted into the 63 kDa species, whereas GRK6-RDD was not. Overepression of GRK6-RDD failed to alter the agonist-mediated phosphorylation of C5aR. Taken together, the results suggest that C5aR is not a substrate for either GRK2 or GRK6 and that GRK6 is very likely autophosphorylated on Ser^{484} and Thr^{485} *in vivo.*

Key Words: **C5a receptor; CD88; phosphorylation; chemoattractant; GRK; autophosphorylation.**

Many studies with receptors that couple to either the adenylate cyclase or the phospholipase C (PLC) pathways suggest that agonist-mediated receptor phosphorylation is a common mechanism by which signaling through G protein-coupled receptors is rapidly attenuated. This process, termed desensitization, involves cellular protein kinases that have yet to be determined for most G protein-coupled receptors. The best studied receptor is the β_2-adrenergic receptor which appears to be phosphorylated by multiple protein kinases, including the cAMP-dependent protein kinase (PKA) and a specific class of kinases termed G protein-coupled receptor kinases (GRK) (1, 2). This latter family comprises six members, (GRK1-6), which appear to be differentially regulated and targeted to the plasma membrane through a variable COOH-terminal region that flanks the catalytic domain (3). All GRKs interact with phospholipids via either a farnesyl group (GRK1) (4), a palmitoyl group (GRK4 and GRK6) (5, 6), a polybasic region (GRK5) (7, 8) or a pleckstrin homology domain (PH domain) that binds to phosphatidyl inositol 4,5-bisphosphate (PIP2) and to the $\beta\gamma$ subunits of G proteins (GRK2 and GRK3) (9–11). This latter interaction increases the kinase activity *in vitro* (12) and may be involved in targeting GRK2 and GRK3 in the close vicinity of activated receptors *in vivo* (13). In addition, two GRKs appear to be differentially regulated by a rapid autophosphorylation. While autophosphorylation reduces the affinity of GRK1 for phosphorylated rhodopsin (14), it appears to increase the ability of GRK5 to phosphorylate the β_2-adrenergic receptor *in vitro* (7).

The use of recombinant receptors reconstituted in liposomes has revealed that purified recombinant GRKs have the unique property to recognize and phos-

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phorylate the visual light receptor rhodopsin as well as β_2-adrenergic, m_2-muscarinic cholinergic, substance P, and α_2-adrenergic receptors in an agonist-dependent manner (8, 12, 15–19). The receptor substrate specificity in *in vitro* assays appears to vary from kinase to kinase. For instance, GRK6 has a weaker ability than GRK2 and GRK5 to phosphorylate the β_2-adrenergic receptor and rhodopsin (17).Moreover, the receptor substrate specificity of GRKs defined in *in vitro* assays may not reflect exactly receptor-kinase interactions *in vivo*. This is suggested by a recent study indicating that mutation of the residues identified as the GRK2 and GRK5 phosphorylation sites on the β2-adrenergic receptor *in vitro* has no effect on the phosphorylation and desensitization of this receptor *in vivo* (20).

Within the family of G protein-coupled receptors, the C5a anaphylatoxin receptor (C5aR) belongs to a subgroup of receptors that mediate chemotaxis and activation of myeloid cells during inflammatory and immune responses (21). The kinases involved in the agonist-dependent phosphorylation of C5aR remains to be identified. Both GRK2 and GRK6 are expressed in myeloid cells and may therefore be involved in the phosphorylation process (22, 23). To determine whether the agonist-occupied C5aR could be a substrate of these kinases, the C5aR and wild-type kinases or dominant negative forms of both GRK2 and GRK6 were transiently coexpressed in COS-7 cells. The results show that neither the wild-type kinases nor their dominant negative forms significantly modify the level of agonist-dependent phosphorylation of C5aR. In addition, we provide strong evidence that GRK6 is autophosphorylated in COS-7 cells.

EXPERIMENTAL PROCEDURES

Materials. Cell culture media and fetal calf serum were purchased from Gibco BRL. Carrier-free [^{32}P] orthophosphoric acid and Na^{125}I were from Amersham (UK). Protein A-Sepharose 4B was from Pharmacia Biotech. Human recombinant C5a, bovine serum albumin (BSA), leupeptin, benzamidine, pepstatin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), p-nitrophenylphosphate were from Sigma Chemicals (St Louis, MO). Restriction endonucleases, Pwo DNA polymerase, other molecular biology reagents, and 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) were purchased from Boehringer-Mannheim. SeeBlue prestained markers were from Novex. Oligonucleotides for mutagenesis were purchased from Genosys Biotechnologies (UK).

Mutagenesis and expression. Mutations in the cDNAs encoding human GRK2 and GRK6 were introduced by site directed mutagenesis, according to the pSELECT-1 mutagenesis protocol from Promega, or by PCR as previously described (24). The coding sequence of the human GRK2 was excised from the pBJIneo vector provided by Antonio De Blasi (Mario Negri, Italy) and inserted between *Hind* III and *Xba* I in pSELECT-1. The mutant primer 5′-GTACGCCATGAGGTGCCTGGAC-3′ (Arg codon is underlined) complementary of the coding strand was used to replace Lys^{220} with an arginine residue (GRK2-R). The mutation was verified by DNA sequencing. The mutated sequence was further digested by *Xba* I and *Mlu* I and used to replace the corresponding fragment in the expression vector pBJIneo.

The coding sequence of GRK6 was excised from the plasmid pBC12BI by *Nsi* I and *Bam* HI and inserted into pSELECT-1 cleaved with *Pst* 1 and *Bam* HI. The mutant primer 5′-CTCTAG-CTTCCTGCAGGCATACAT-3′ (Arg codon is underlined) was used to replace Lys^{215} with an arginine residue (GRK6-R). pBC12BI-GRK6 was cleaved with *Bst* XI and *Bgl* II and the 0.9 kb fragment was replaced by the corresponding sequence containing the desired mutation. To replace Ser^{484} and Thr^{485} with aspartate residues in GRK6-R the sense primer 5′-GGATGTTCTGGACATTGAACAG-TTCGATGATGTCAAGGGCGTGGAGCTGG-3′ (Asp codons are underlined) was used with the antisense primer 5′-CACCAGCAG-TTGGCGGTAGCAGGATCCGCG-3′ (primer A) to amplify a 346 bp fragment of GRK6 cDNA. The sense primer 5′-GCTCTAGAGC-AGAAGGCTTCATTTGGTGACCAAGC-3′ (primer B) was used with the antisense primer 5′-CTCCAGCTCCACGCCCTTGACATCATC-GAACTGTTCAATGTCCAG (Asp codons are underlined) to generate a 1490 bp fragment of pGRK6-R. Aliquots of the resulting PCR fragments were mixed and used in a third PCR with primers A and B to generate a 1800 bp fragment. The PCR fragment was cleaved by *Xba* I and *Bam* HI and cloned in pCDNA3.1 (−) (GRK6-RDD). A kinase mutated exclusively at the level of Ser^{484} and Thr^{485} (GRK6-DD) was generated by replacing the *Bgl* II-*Bam* HI fragment in the wild-type kinase by the corresponding fragment from GRK6-RDD in the vector pBC12BI.

Transfection of COS-7 cells. COS-7 cells were grown and transfected by electroporation as described previously (25). The cDNA encoding human wild-type GRK2 was in pBJIneo, that encoding human GRK6, GRK6-R or GRK6DD was in pBC12BI, and that encoding human GRK2-R or GRK6-RDD was in pCDNA3.1. Cells were electroporated with a Bio-Rad gene pulser using a pulse of 0.3 kV for 10 ms. Sixty to seventy-two hours after transfection, cells were used for [^{32}P] orthophosphoric acid metabolic labeling and binding of ^{125}I labeled C5a.

Radioligand binding. Human recombinant C5a was labeled with ^{125}I as described previously (25). All binding studies were carried out at 4°C with subconfluent COS-7 cell monolayers three days after transfection as described previously (26). Non-specific binding was determined by the addition of a 20-fold excess of unlabeled C5a.

Immunoblotting. Cell monolayers were lysed in 100 μl of 2-fold Laemmli sample buffer supplemented with 10 mM dithiothreitol and briefly sonicated with a microtip. Proteins were then separated by SDS–PAGE and transferred to a 0.22 μm Protran nitrocellulose filter (Schleicher & Schüell). After transfer, the filter was incubated in PBS containing 0.1% Tween 20 and 3% BSA for 1 hour at room temperature and then overnight in the same solution containing antibodies. IgGs to GRK6 were affinity-purified from the serum of a rabbit immunized against the C-terminal peptide YGNCSDSEEELPTRL-COOH coupled to ovalbumin as previously described (26). Filters were extensively washed with PBS containing 0.1% Tween 20, and incubated with ^{125}I-labeled protein A (1 μCi/ml of PBS/Tween 20/BSA) for 2 hours, at room temperature. After washing filters in PBS/Tween 20, the bound radioactivity was visualized by autoradiography using Fuji RX film at −80°C. Quantification was performed with a Molecular Dynamics phosphorImager.

Metabolic labeling and immunoprecipitation. Transiently transfected COS-7 cells were metabolically labeled with [^{32}P] orthophosphoric acid (0.3 -0.5 mCi/ml) as described previously (26). Phosphorylation of C5aR was initiated with 50 nM C5a. After 15 min incubation at 37°C, cells were lysed in 1 ml of ice-cold RIPA buffer, C5aR was immunoprecipitated and analyzed by SDS–PAGE under reducing conditions and autoradiography as described previously (27). To be able to compare the level of phosphorylation of C5aR, the volume of cell lysates withdrawn for immunoprecipitation was adjusted so that the same amount of surface-expressed receptor was immunoprecipitated from plate to plate.

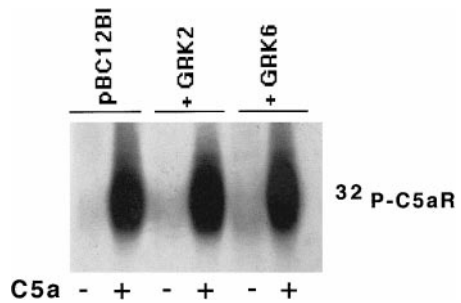

FIG. 1. The agonist-mediated phosphorylation of C5aR in COS-7 cells is not significantly enhanced by the coexpression of wild-type GRK2 and GRK6. COS-7 cells were cotransfected with C5aR and wild-type kinases. Control cells were cotransfected with C5aR and an empty vector (pBC12BI). Three days after transfection, cell surface expressed receptors were quantified for each condition of transfection. In companion plates, C5aR phosphorylation was initiated by the addition of 50 nM C5a after prelabeling with [^{32}P] orthophosphoric acid. After 15 min of stimulation with C5a, cells were lysed and aliquots corresponding to the same number of surface-expressed receptors were processed for immunoprecipitation of C5aR. The immunoprecipitated material was submitted to SDS–PAGE and autoradiography on Fuji RX films. COS-7 cells were cotransfected with C5aR and wild-type GRK2 and GRK6 cDNAs. Control cells were cotransfected with the appropriate empty vector. The figure is representative of two independent experiments.

RESULTS AND DISCUSION

The agonist-mediated phosphorylation of the C5a receptor is not enhanced by overexpression of wild-type GRK2 and GRK6. The agonist-dependent phosphorylation of C5aR has been demonstrated in myeloid cells as well as in COS-7 cells and phosphorylation sites have been identified on all serine residues of the cytoplasmic tail (25, 27). The nature of the kinase(s) involved in this process remains unknown. Although recent studies have shown that GRK2 and GRK6 are expressed in myeloid cells, it is not known whether they play a role in the phosphorylation of the C5aR.

Neither GRK2 nor GRK6 were immunodetected in lysates of COS-7 cells but they might be present at a level below the threshold of detection with antipeptide antibodies and ^{125}I-labeled protein A (not shown). However, COS-7 cells appear to be equipped with kinases (GRKs or other kinases) that efficiently phosphorylate the C5aR. If either GRK2 or GRK6 is involved in the agonist-induced phosphorylation of C5aR, an overexpression of either of them should potentiate the phosphorylation of the receptor. In the case of other receptors, such as the α_{1B}–adrenergic receptor (28) and the chemokine receptor CCR5 (29), the agonist-induced phosphorylation was found to be enhanced when wild-type GRK2 is coexpressed with the receptor. In contrast to these receptors, the agonist-mediated phosphorylation of C5aR was not enhanced by overexpression of either GRK2 or GRK6 (Fig. 1). Incidentally, we consistently observed a higher expression of C5aR (about 2-fold) when GRK6 was transiently coexpressed with the receptor (Boulay, unpublished data). However, it is presently not known if GRK6-induced upregulation of C5aR occurs in cell lines with endogenous C5aR expression and is of physiological significance.

Overexpression of a dominant negative mutant of GRK2 or GRK6 has no effect on the agonist-mediated phosphorylation of C5aR. If the C5aR were a good substrate for GRK2 or GRK6, the expression of a dominant negative mutant should compete with the endogenous kinase(s) and result in an inhibition of the C5a-induced phosphorylation. Protein kinases contain, in the subdomain II of the catalytic domain, an universally conserved lysine residue involved in the phosphotransfer reaction (30). In GRK2, substitution of the invariant Lys^{220} by an arginine results in a mutant devoid of kinase activity but able to inhibit the agonist-induced phosphorylation of β_2-adrenergic, angiotensin II, α_{1B}-adrenergic, and δ-opioid receptors in transfected cells (31–33). Likewise, we replaced the equivalent Lys^{215} of GRK6 by an arginine (GRK6-R). However, while it is clear from *in vitro* and cell transfection studies that GRK2-R behaves as a dominant negative mutant, it is not established that the mutation K215R in GRK6 yields a dominant negative mutant. Indeed, GRK6 is highly homologous to GRK5 which has been shown to be autophosphorylated on Ser^{484} and Thr^{485}. In the case of GRK5, it has been shown that an active GRK5 mutant, which lacks phosphoamino acids at positions 484 and 485, has a 15-20-fold reduced ability to phosphorylate the β2-adrenergic receptor and rhodopsin compared to the wild-type kinase (7). As GRK5, GRK6 possesses the putative autophosphorylation motif DIEQFSTVKG (residues 479 to 488) but is poorly autophosphorylated *in vitro* (17). However, one cannot exclude the possibility that GRK6 is autophosphorylated in a cellular context. Autophosphorylation might be required for the efficient interaction of GRK6 with its substrate(s). By essence, GRK6-R is unable to be autophosphorylated and may behave as a dead kinase rather than a dominant negative kinase. A mutant that mimics a possible autophosphorylated state was therefore constructed by replacing Ser^{484} and Thr^{485} by aspartic acid residues in GRK6-R, yielding the mutant kinase GRK6-RDD.

To examine if the overexpression of the mutant kinases inhibited the C5a-induced phosphorylation of C5aR, we transiently coexpressed either GRK2-R, GRK6-R, or GRK6-RDD with the receptor in COS-7 cells. The same number of C5a-stimulated receptors was immunoprecipitated and the level of radioactivity incorporated in the receptor was determined after electrophoresis on polyacrylamide gel. No significant difference in the level of phosphorylation could be observed with either of the three mutant kinases (Fig. 2).

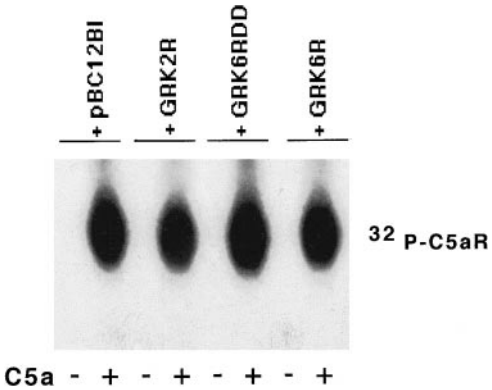

FIG. 2. Overexpression of GRK2-R, GRK6-R, and GRK6-RDD fails to inhibit the agonist-mediated phosphorylation of C5aR in COS-7 cells. COS-7 cells were cotransfected with C5aR and mutant kinases (GRK2-R, GRK6-R, or GRK6-RDD). Control cells were cotransfected with C5aR and an empty vector (pBC12BI). Metabolic labeling and C5a stimulation were performed as described in the legend to Fig. 1. The immunoprecipitated material was submitted to SDS–PAGE and autoradiography on Fuji RX films. The figure is representative of two independent experiments.

Altogether the results suggest that C5aR is not a good substrate for GRK2 and GRK6.

Evidence that GRK6 is autophosphorylated in COS-7 cells. Although Loudon and coworkers have shown that recombinant GRK6 purified from Sf9 cells is very poorly autophosphorylated in an *in vitro* assay (17), one cannot exclude the possibility that GRK6 behaves differently in a cellular context. Indeed, the following experiments provide strong evidence that GRK6 is autophosphorylated in COS-7 cells.

The wild-type GRK6 and the different GRK6 mutants shown in Fig. 3A were expressed in COS-7 cells. When the recombinant proteins were analyzed by immunoblotting after direct lysis of the cells in Laemmli sample buffer, different patterns of electrophoretic mobility were observed. Wild-type GRK6 migrated as a doublet of 63 kDa and 66 kDa (Fig. 3B, left panel). Neither the 63 kDa species nor the 66 kDa species was detected in cells transfected with an empty expression vector, indicating that both bands were related to GRK6. Only the 63 kDa species could be detected in lysates of cells transfected with GRK6-R (Fig. 3B, left panel), suggesting that GRK6 undergoes a post-translational modification which is dependent on the phosphotransfer reaction catalyzed by Lys^{215}.

This latter observation is reminiscent of the behavior of GRK5 which also migrates as a doublet after autophosphorylation or as the fast moving band of this doublet when ATP is absent or when the invariant Lys^{215} is replaced by an arginine (34). The retarded electrophoretic migration of GRK5 appears to result from phosphorylation of Ser^{484} and Thr^{485}. A GRK5 mutant, in which the replacement of Ser^{484} and Thr^{485} by an aspartic acid residue mimics phosphoserine or phosphothreonine, was found to migrate with the same electrophoretic mobility as autophosphorylated GRK5 (34). Likewise, when lysates from cells transfected with mutant GRK6 carrying the corresponding mutations (GRK6-DD and GRK6-RDD) were analyzed by immunoblotting, both mutant proteins were found to migrate as a single species of 66 kDa (Fig. 3B, right panel). This result strongly suggests that the 66 kDa band represents autophosphorylated GRK6.

Unfortunately, the rabbit polyclonal antibody used in this study as well as another antibody directed to residues 391 to 408 in GRK6 had no ability to immunoprecipitate GRK6. Therefore, we were unable to demonstrate the incorporation of phosphate in GRK6 after metabolic labeling with [^{32}P] orthophosphoric acid. We made the assumption that, if the 66 kDa readily represents an autophosphorylated form of GRK6, the protein phosphatases that are present in cell extracts should rapidly induce the conversion of the 66 kDa band into a single species with an apparent mass of 63 kDa. Conversely, inhibition of serine and threonine protein phosphatases should block this conversion. As shown in Fig. 4A (left panel), lysis of cells in a non-denaturing buffer without phosphatase inhibitors yielded exclusively a 63 kDa protein, whereas a major species of 66 kDa and a minor species of 63 kDa were recovered in the presence of protein phosphatase

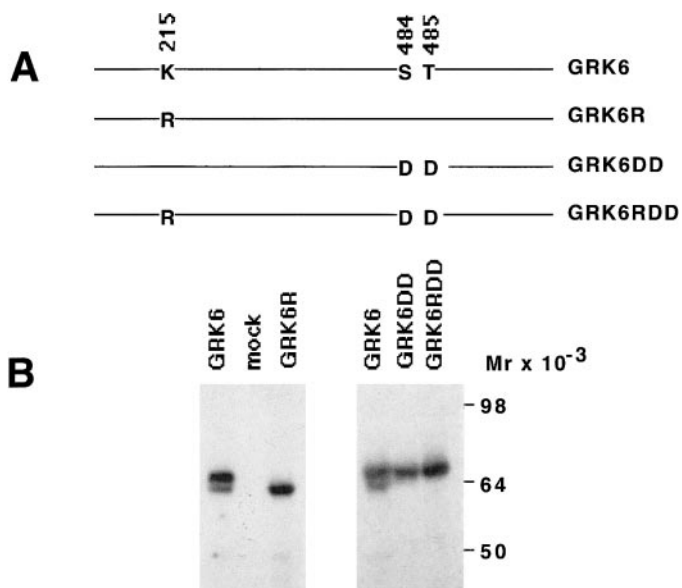

FIG. 3. Western blot analysis of wild-type and mutant forms of GRK6. (A) Positions of the mutated residues in the primary sequence of GRK6. (B) COS-7 cells were transfected with either an empty vector pBC12BI (mock), or a vector encoding GRK6, GRK6-R, GRK6DD, or GRK6-RDD as described under "Experimental Procedures." After three days, cells were directly lysed in 2-fold Laemmli sample buffer under reducing conditions and samples were analyzed by immunoblotting with affinity-purified anti-GRK6 IgGs. Bound primary IgGs were detected with ^{125}I-labeled Protein A and autoradiography on Fuji RX films.

inhibitors. The possibility that the 63 kDa GRK6 species could arise from a limited proteolysis of the N-terminal region is very unlikely as revealed by the following control experiment. The mutant protein GRK6-RDD was not converted into a fast moving species, even after a prolonged exposure at 37°C, in the absence of protein phosphatase inhibitors (Fig. 4A, right panel). In addition, the conversion of the 66 kDa band to the 63 kDa species was partially prevented by okadaic acid for concentrations superior to 100 nM (Fig. 4B). Given the lack of effect of okadaic acid for concentrations lower than 100 nM, the protein phosphatase 2A is unlikely to be the major serine/threonine protein phosphatase that controls the state of phosphorylation of GRK6. Inhibition of PP2A is indeed known to occur *in vitro* at subnanomolar concentrations of okadaic acid (35).

To evaluate the time course of conversion of the 66 kDa band to the 63 kDa species, non-denatured cell lysates were incubated for various period of time, at 37°C, in the absence of phosphatase inhibitors. The ratio of conversion was assessed by immunoblotting analysis. Quantification of the bound ^{125}I-labeled protein A indicated that this conversion was half-maximal in less than one minute (Fig. 5).

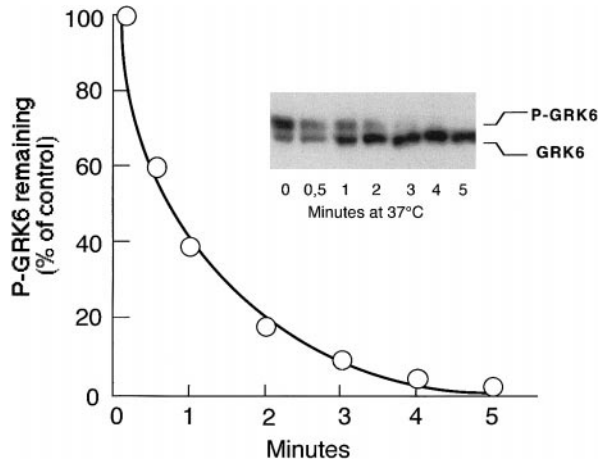

FIG. 5. Kinetics of GRK6 dephosphorylation *in vitro*. COS-7 cells were transfected with GRK6 and lysed in 1% Triton X-100 buffer in the absence of phosphatase inhibitors. Cell extracts were incubated at 37°C. At the indicated time points aliquots were removed, diluted in 4-fold Laemmli sample buffer and processed for detection of GRK6 by immunoblotting. The rate of conversion of the 66 kDa species into a 63 kDa species was calculated after quantification with a phosphorImager. The figure is representative of two independent experiments.

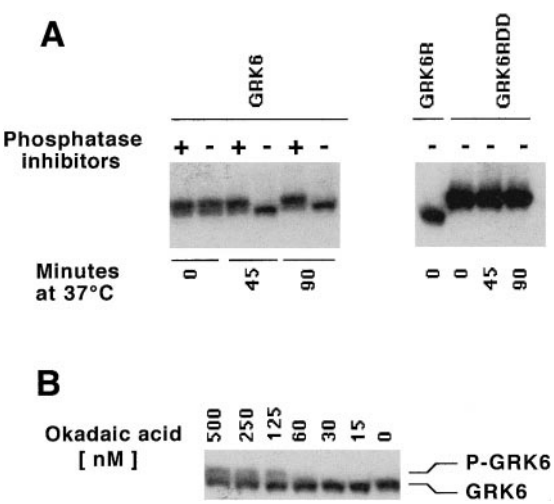

FIG. 4. Phosphatase inhibitors inhibit the mobility shift of GRK6. (A) Left panel: COS-7 cells transfected with wild-type GRK6 were lysed in 1% Triton X-100 in the absence or presence of phosphatase inhibitors (2 μM cyclosporine A, 1 μM okadaic acid, 60 nM FK506, 10 mM NaF, 250 μM Na_3VO_4). Lysates were incubated for 0, 45, or 90 min at 37°C. Right panel: lysates of cells expressing either GRK6-R or GRK6-RDD were incubated, at 37°C, in the absence of phosphatase inhibitors for various periods of time. At the end of the indicated periods, lysates were further diluted with 4-fold Laemmli sample buffer and analyzed by immunoblotting with affinity-purified anti-GRK6 IgGs and ^{125}I-labeled Protein A. (B) COS-7 cells transfected with wild-type GRK6 were lysed in Triton X-100 supplemented with various concentrations of okadaic acid and analyzed by immunoblotting. The conversion of the 66 kDa form of GRK6 into a 63 kDa species was inhibited when the concentration of okadaic acid was higher than 60 nM. The figure is representative of three independent experiments.

Taken together the data strongly suggest that GRK6, like GRK5, is autophosphorylated on Ser^{484} and Thr^{485}. Based on the relative intensity of the two bands, it appears that about 70% of GRK6 molecules are maintained in an autophosphorylated state in COS-7 cells.

CONCLUSION

Altogether the results suggest that the C5aR is not a good substrate for GRK2 or GRK6. This conclusion is consistent with the observation that, in leukocytes, C5a is unable to induce the translocation of cytosolic GRK2 to the plasma membrane (22). Translocation of GRK2 has been shown to be facilitated by interactions with released $G_{\beta\gamma}$ subunits and is therefore considered as an essential step in GRK2-mediated homologous desensitization (36, 37). The patterns of electrophoretic mobility of the different GRK6 mutants strongly suggest that autophosphorylation does occur on Ser^{484} and/or Thr^{485}, making GRK6 much more similar to GRK5 than previously thought. Whether the autophosphorylation of GRK6 modulates its activity remains however to be established.

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