Identification of G Protein-Coupled Receptor Kinase 2 Phosphorylation Sites Responsible for Agonist-Stimulated δ-Opioid Receptor Phosphorylation

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ABSTRACT

Agonist-induced receptor phosphorylation is an initial step in opioid receptor desensitization, a molecular mechanism of opioid tolerance and dependence. Our previous research suggested that agonist-induced δ-opioid receptor (DOR) phosphorylation occurs at the receptor carboxyl terminal domain. The current study was carried out to identify the site of DOR phosphorylation during agonist stimulation and the kinases catalyzing this reaction. Truncation (Δ15) or substitutions (T358A, T361A, and S363G single or triple mutants) at the DOR cytoplasmic tail caused 80 to 100% loss of opioid-stimulated receptor phosphorylation, indicating that T358, T361, and S363 all contribute and are cooperatively involved in agonist-stimulated DOR phosphorylation. Coexpression of GRK2 strongly enhanced agonist-stimulated phosphorylation of the wild-type DOR (WT), but Δ15 or mutant DOR (T358A/T361A/S363G) failed to show any detectable phosphorylation under these conditions. These results demonstrate that T358, T361, and S363 are required for agonist-induced and GRK-mediated receptor phosphorylation. Agonist-induced receptor phosphorylation was severely impaired by substitution of either T358 or S363 with aspartic acid residue, but phosphorylation of the T361D mutant was comparable with that of WT. In the presence of exogenously expressed GRK2, phosphorylation levels of T358D and S363D mutants were approximately half of that of WT, whereas significant phosphorylation of the T358/S363 double-point mutant was not detected. These results indicate that both T358 and S363 residues at the DOR carboxyl terminus are capable of serving cooperatively as phosphate acceptor sites of GRK2 in vivo. Taken together, we have demonstrated that agonist-induced opioid receptor phosphorylation occurs exclusively at two phosphate acceptor sites (T358 and S363) of GRK2 at the DOR carboxyl terminus. These results represent the identification of the GRK phosphorylation site on an opioid receptor for the first time and demonstrate that GRK is the prominent kinase responsible for agonist-induced opioid receptor phosphorylation in vivo.

Opioid receptors are G protein-coupled receptors (GPCRs) including μ-, δ-, and κ-subtypes. Interaction of opiates with opioid receptors produces a strong analgesic effect, but chronic use of opioid drugs causes tolerance and dependence and thus limits the clinical application and results in opioid abuse. The molecular mechanisms underlying opioid tolerance and dependence are complex and not well understood, but desensitization of the opioid receptor has been implicated as a possible mechanism (Nestler and Aghajanian, 1997). Studies of adrenergic receptors and rhodopsin show that mechanisms of desensitization of many GPCRs include phosphorylation of agonist-occupied receptor, binding of arrestin proteins specifically to the phosphorylated receptor, subsequent receptor sequestration, and other agonist- or G protein-independent events (Schwinn et al., 1992; Ferguson et al., 1996; Palczewski and Saari, 1997; Krupnick and Benovic, 1998).

Phosphorylation of GPCRs has been considered an initial step in acute receptor desensitization triggering receptor/G protein uncoupling and involves GPCR kinases (GRKs) and second messenger-activated protein kinases (PKA and PKC; Schwinn et al., 1992). Studies show that chronic opiate treatment strongly increases GRK levels and PKC activity in specific brain regions, and inhibition of PKC activity attenuates the development of morphine tolerance (Terwilliger et al., 1994; Mao et al., 1995; Mayer et al., 1995). Our previous

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ABBREVIATIONS: GPCR, G protein-coupled receptor; DOR, δ-opioid receptor; DPDPE, [D-Pen[2],D-Pen[5]]enkephalin; GRK, G protein-coupled receptor kinase; HEK, human embryonic kidney; PKA, protein kinase A; PKC, protein kinase C; WT, wild type δ-opioid receptor; MOR, μ-opioid receptor; KOR, κ-opioid receptor; HA, hemagglutinin; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; DAMGO, [D-Ala[2],N-Me-Phe[4], Gly[5]-ol]enkephalin.

research and that of other groups indicate that activation of PKC induces phosphorylation of δ-opioid receptors (DORs) and μ-opioid receptors (MORs) in an agonist-independent manner and enhances receptor desensitization (Pei et al., 1995; Zhang et al., 1996; Zhao et al., 1997). Inhibition of PKC activity strongly attenuates opioid receptor desensitization (Cai et al., 1996; Zhang et al., 1996; Pei et al., 1997). However, inhibition of PKC activity or depletion of PKC fails to block agonist-induced phosphorylation of the DOR (Pei et al., 1995). Phosphorylation of DOR, MOR, and KOR in response to agonist stimulation has been observed by us and those from a number of other laboratories (Arden et al., 1995; Pei et al., 1995; Appleyard et al., 1997). Accumulating evidence indicates a primary role of GRKs in agonist-induced phosphorylation and homologous desensitization of opioid receptors. Studies on DOR, MOR, and KOR reveal that overexpression of GRK2 enhances agonist-dependent receptor phosphorylation and desensitization and overexpression of a dominant-negative mutant of GRK2 or inhibition of GRK activity blocks desensitization of opioid receptors (Raynor et al., 1994; Pei et al., 1995; Kovoor et al., 1997; Appleyard et al., 1999). The abilities of opioid agonists to induce MOR desensitization are related to their abilities to promote GRK-dependent phosphorylation (Kovoor et al., 1998; Zhang et al., 1998). These results suggest that GRKs are important mediators in agonist-induced opioid receptor phosphorylation and desensitization.

Desensitization studies of opioid receptors with site-directed mutagenesis methods suggest that the agonist-stimulated phosphorylation site is located in the portion of carboxyl terminus of the opioid receptor (Kovoor et al., 1997; Pak et al., 1997; Burd et al., 1998; Appleyard et al., 1999). However, studies to identify the agonist-dependent phosphorylation site in opioid receptors by directly measuring the receptor phosphorylation are lacking. The significance and mechanism of GRK- and other protein kinase- mediated receptor phosphorylation on regulation of opioid signaling remains to be demonstrated directly. Our previous studies demonstrated that agonist-induced DOR internalization and subsequent receptor/β-arrestin interaction require the DOR carboxyl terminus (Trapaidze et al., 1996; Cheng et al., 1998) and the agonist-stimulated and GRK-mediated DOR phosphorylation sites are located at its carboxyl terminus, which contains no Tyr but six Ser and Thr residues as potential phosphorylation sites (Zhao et al., 1997). In the current study, we have identified the amino acids acting as phosphate acceptors in the agonist-induced DOR phosphorylation, and we have demonstrated that agonist-induced opioid receptor phosphorylation occurs exclusively at two GRK sites close to DOR carboxyl terminus.

Materials and Methods

Construction of Mutants. Plasmids encoding influenza hemagglutinin (HA)-tagged mouse wild-type DOR (WT) and the carboxyl-terminal 31-residue-truncated DOR (Δ31) were constructed in pcDNA3 (Stratagene, La Jolla, CA) as described previously (Pei et al., 1995; Zhao et al., 1997). S344G, T352A, T353A, T358A, T361A, and Δ15 with an HA-tag at the N terminus were constructed by exchanging the *NotI/XbaI* fragment of WT with the corresponding fragment in FLAG-tagged mutant DORs (Trapaidze et al., 1996). The HA-tagged S363G, M3, T358D, T361D, S363D, and D2 were

constructed by polymerase chain reaction mutagenesis, and the authenticity of the sequence was confirmed by DNA sequencing.

Transfection. Human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Rockville, MD) were plated in 60-mm tissue culture dishes at 1×10^6 cells/dish in minimum essential medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum and transfected 20 h later with 4 μg of plasmid by the calcium phosphate-DNA coprecipitation method (Cheng et al., 1998). In some experiments, 3.5 μg of bovine GRK2 cDNA was cotransfected with DOR cDNA. The cells were harvested and used 48 h after transfection. Receptor expression was measured with the [³H]diprenorphine-binding assay (Amersham Pharmacia Biotech, Piscataway, NJ) as described (Zhao et al., 1997). To ensure quantitative determination of receptor functions, expression levels of the WT and mutant DORs were kept at 3 pmol/mg protein with a fluctuation of less than 15% by carefully controlling cell culture and transfection conditions.

Immunofluorescence Analysis. The cells grown on coverslips were transfected with WT or mutant DORs. The cells were washed with PBS and incubated for 20 min in 4% polyformaldehyde 48 h after transfection. The cells were then incubated for 1 h at 4°C with 1 μg/ml 12CA5 (an HA-epitope-specific monoclonal antibody from Boehringer Mannheim, Mannheim, Germany) and 45 min with fluorescein-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA), washed with PBS, and mounted on glass slides with 50% glycerol. Scanning images were recorded with a TCS NT laser confocal microscope (Leica Microsystems, Bensheim, Germany).

Immunoprecipitation and Western Blotting. Immunoprecipitation was carried out essentially as previously described (Pei et al., 1995). Briefly, the cells were lysed on ice for 1.5 h in buffer IP containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.1% SDS and 1% Nonidet P-40, 0.5% sodium deoxycholate, 10 mM disodium pyrophosphate, 10 mM sodium fluoride, 0.2 mM phenylmethanesulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml benzamidine. The lysates were centrifuged at 14,000*g* for 30 min. Receptors were then immunoprecipitated with 12CA5 and protein A Sepharose (Amersham Pharmacia Biotech) overnight at 4°C. After the sample was washed in buffer IP, the absorbed complexes were removed from the beads by heating for 20 min at 50°C in reducing SDS-polyacrylamide gel electrophoresis sample buffer and analyzed on 10% polyacrylamide gels. Proteins resolved on gels were transferred to nitrocellulose membranes and detected with antibodies. The HA-tagged receptors were detected with biotinylated 12CA5 and a streptavidin-horseradish peroxidase conjugate (Boehringer Mannheim); GRK2 was detected with rabbit anti-GRK2 antiserum (Pei et al., 1995) and goat anti-rabbit IgG horseradish peroxidase conjugate with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

Receptor Phosphorylation. Measurement of opioid receptor phosphorylation was carried out as described previously (Pei et al., 1995; Zhao et al., 1997). Briefly, the cells were metabolically labeled at 37°C for 60 min with 60 μCi/ml [³²P] P_i (Amersham Pharmacia Biotech) in phosphate-free Dulbecco's modified Eagle's medium (Life Technologies) 48 h after transfection. To inhibit phosphatase, 1 μM okadaic acid or 5 μM cantharidin (Calbiochem, La Jolla, CA) was added 3 min before the end of labeling. Then the cells were exposed to 1 μM [D-Pen², D-Pen⁵]-enkephalin (DPDPE, Sigma) for 10 min at 37°C and solubilized on ice for 1.5 h in buffer IP. The exogenous DORs were immunoprecipitated and analyzed on 10% polyacrylamide gels. The gels were subjected to quantitative analysis with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and autoradiographed to X-ray film after drying.

cAMP Assay. Cells were challenged with 1 μM DPDPE in the presence of 10 μM forskolin (Sigma) and 500 μM 1-methyl-3-isobutylxanthine (Sigma) at 37°C for 15 min, and the cAMP level of each sample was determined with a radioimmunoassay as described previously (Cai et al., 1996). Data were averaged from triplicate samples.

Results

As shown in Fig. 1, the carboxyl-terminal 31 residues of DOR contain two Ser and four Thr residues posing as potential phosphorylation sites in this region. To assess which Ser/Thr residue could act as a phosphate acceptor upon agonist stimulation, truncation mutants of DORs were constructed by removing the last 15 (Δ15) or 31 (Δ31) residues containing the last three or six potential phosphorylation sites, respectively, from the carboxyl terminus of the wild-type receptor (WT, Fig. 1). WT, Δ15, and Δ31 were transiently transfected into HEK293 cells, and their expressions were examined 48 h post-transfection (Fig. 2). Results from immunoprecipitation experiments show that the truncation mutant Δ15 and Δ31 migrated faster than the full-length WT on gel and expressed at levels comparable with that of WT (Fig. 2A). The surface expression of the opioid receptors was analyzed under a laser confocal fluorescence microscope. Scanning images show that WT, Δ15, and Δ31 were expressed on the cell surface in a similar distribution pattern (Fig. 2B). Radioligand-binding experiments indicate that the expression levels of WT, Δ15, and Δ31 were comparable (approximately 3 pmol/mg), consistent with the data obtained from surface immunostaining. Furthermore, $[^{35}S]GTP\gamma S$ binding and cAMP assays demonstrate that truncating the carboxyl terminus of DOR affects neither receptor/G protein coupling (data not shown) nor DOR-mediated inhibition of adenylyl cyclase (Fig. 2C). It has been demonstrated that the carboxyl terminus truncated DOR internalizes, with rapid kinetics and high efficiency similar to that of the WT, and that agonist-stimulated DOR internalization is not dependent on DOR phosphorylation in HEK293 cells (Murray et al., 1998). Consistent with this, our results show that deletion of the DOR carboxyl terminus had no significant effect on receptor internalization induced by DPDPE in HEK293 cells under the conditions used (data not shown).

The functions of the last six and three potential Ser/Thr phosphorylation sites in DOR carboxyl tail were investigated next with the WT and truncated DORs. HEK293 cells transiently expressing WT, Δ15, and Δ31 were metabolically labeled with $[^{32}P]$ P_i, and the extent of receptor phosphoryla-

tion in response to agonist stimulation was determined after immunoprecipitation and phosphor-imaging analysis. As shown in Fig. 3, challenging the cells expressing WT with DPDPE, a specific agonist of DOR, resulted in strong phosphorylation of DOR, and the agonist-induced receptor phosphorylation was abolished completely in the cells expressing Δ31 lacking the last six potential Ser/Thr phosphorylation residues (Fig. 3). Furthermore, shorter truncation (Δ15) that preserves S344, T352, and T353 in the carboxyl terminus failed to restore DPDPE-stimulated receptor phosphorylation (Fig. 3), indicating the requirement of the last 15 residues containing potential phosphorylation sites T358, T361, and S363 in the receptor carboxyl tail for the agonist-induced phosphorylation.

To confirm the result obtained with deletion mutants and to minimize possible disruption on the secondary structure of the receptor cytoplasmic tail, a triple point mutant of DOR with the three potential phosphorylation residues proximal to the carboxyl terminus eliminated by substitution mutation (T358A/T361A/S363G, designated M3) and single point mutants (S344G, T352A, T353A, T358A, T361A, and S363G) with a single potential phosphorylation site eliminated were constructed (Fig. 1). These substitution mutations did not affect receptor surface expression, ligand binding, or G pro-
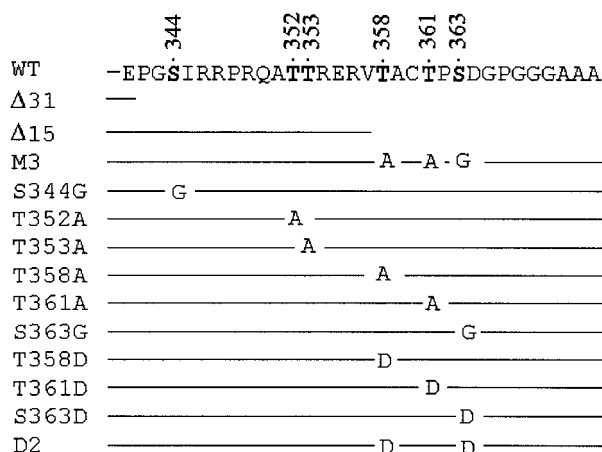

Fig. 2. Analysis of expression and function of WT, Δ31, and Δ15 by immunofluorescence, immunoprecipitation, and cAMP assay. HEK293 cells transiently expressing WT, Δ31, and Δ15 were analyzed 48 h after transfection. A, untransfected cells (Control) and the cells transiently expressing WT, Δ31, and Δ15 were lysed and immunoprecipitated with 12CA5. The HA-tagged DORs were detected with biotinylated 12CA5 and streptavidin-peroxidase conjugate after blotting. B, immunofluorescence staining of the receptors was done with 12CA5 and goat anti-mouse Ig-fluorescein conjugate. Cells were imaged by laser confocal fluorescence scanning microscope. Representative confocal optical sections imaged through the center of transfected cells are shown as insets. C, cells were incubated with or without 1 μM DPDPE for 15 min, and the cAMP levels were determined. **$P < .01$ compared with untreated control. The data are means ± S.E. of three independent experiments.

Fig. 1. Schematic representation of the carboxyl-terminal tail sequences of the WT and the mutant DORs. The carboxyl-terminal residues 341 to 372 of the mouse WT are shown in single-letter amino acid codes, with the potential phosphorylation Ser and Thr residues in bold letters. The amino acid residue substitutions in the mutant DORs are as indicated.
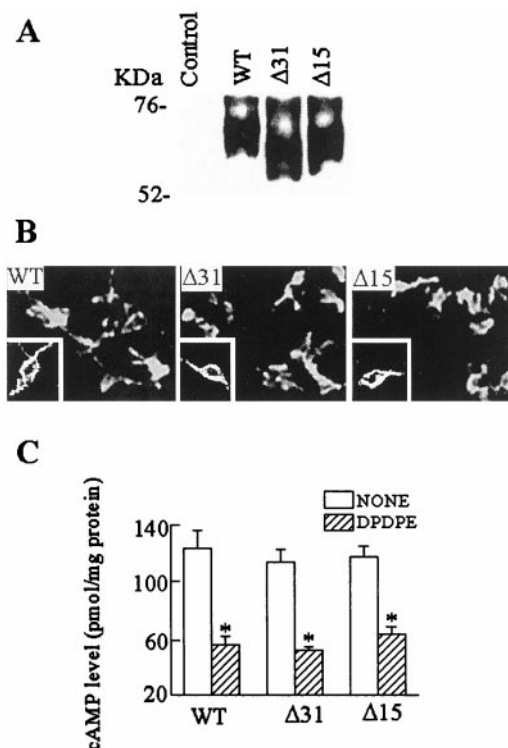
tein coupling (data not shown). No significant phosphorylation of M3, which contains only S344, T352, and T353 as potential phosphoacceptor sites, was observed after DPDPE stimulation (Fig. 3). This agrees with the result obtained with Δ15 and indicates the presence of agonist-dependent receptor phosphorylation site(s) among T358, T361, and S363 of the DOR.

To explore whether some or all of the three potential Ser/Thr phosphorylation sites are necessary for the agonist-induced receptor phosphorylation, DPDPE-induced phosphorylation of mutant DORs with putative phosphoacceptor T358, T361, or S363 individually knocked-out by single point substitution (T358A, T361A, and S363G) was determined in HEK293 cells. As shown in Fig. 4, single point substitution of potential phosphoacceptor T358, T361, or S363 with an Ala or Gly resulted in more than 80 to 90% loss in agonist-induced DOR phosphorylation, whereas mutating S344, T352, or T353 residues distal from the carboxyl terminus had no significant effect. These data support the notion that the agonist-induced phosphorylation occurs at residues among the last three potential Ser/Thr phosphorylation sites in the DOR carboxyl terminus and indicate that T358, T361, and S363 all contribute to and are co-operatively involved in agonist-induced DOR phosphorylation.

Analysis of the carboxyl-terminal sequence of mouse DOR revealed that 1) T358, T361, and S363 do not reside in a typical phosphorylation site context for PKC, PKA, or CaMK and 2) two putative GRK phosphorylation sites are present (Onorato et al., 1991). Our research shows that activation of PKA or inhibition of PKC, mitogen-activated protein kinase, or CaMK activities had no significant effect on agonist-induced phosphorylation of DOR (data not shown). These results are consistent with the results obtained by Pei et al. (1995) that neither PKC nor PKA is involved in agonist-induced DOR phosphorylation and GRKs are the principal kinases involved (Pei et al., 1995; Hasbi et al., 1998). Our earlier research demonstrated that agonist-stimulated phosphorylation mediated by GRK occurs at the carboxyl terminus of DOR (Zhao et al., 1997). To identify Ser/Thr residues involved in GRK-mediated receptor phosphorylation, the effect of overexpression GRK2 on DPDPE-stimulated phosphorylation of WT, Δ31, Δ15, and M3 was examined in HEK293 cells. As shown in Fig. 5, transfection of GRK2 resulted in an approximately 20-fold increase in GRK expression and strongly enhanced DPDPE-stimulated phosphorylation of WT, but Δ31, Δ15, or M3 failed to show any detectable phosphorylation under the same conditions. Results similar to those presented in Figs. 2 to 5 were obtained with
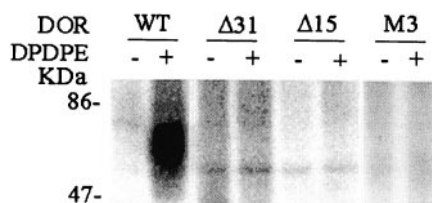
[D-Ala²,D-Leu⁵]enkephalin, another specific agonist of DOR, and NG108–15 cells expressing endogenous DOR (data not shown). Furthermore, our results show that coexpression of GRK2 resulted in a remarkable reduction of opioid-induced, DOR-mediated inhibition of cellular cAMP accumulation, whereas eliminating T358, T361, or S363 of DOR by truncation strongly attenuated inhibition of GRK on the responsiveness of DOR under the same conditions (data not shown). These results clearly demonstrate that agonist-stimulated GRK phosphorylation site(s) is among T358, T361, and S363, the last three potential phosphorylation sites in the DOR carboxyl terminus.

T358, T361, and S363 could be involved in DOR phosphorylation by serving as a GRK phosphoacceptor site or contributing to the interaction of the receptor and GRK upon agonist stimulation. The impairment of receptor phosphorylation after single substitutions of these three residues with an Ala or Gly shown in Fig. 4 may be a result of either removal of a GRK phosphorylation site or disruption of the receptor-GRK
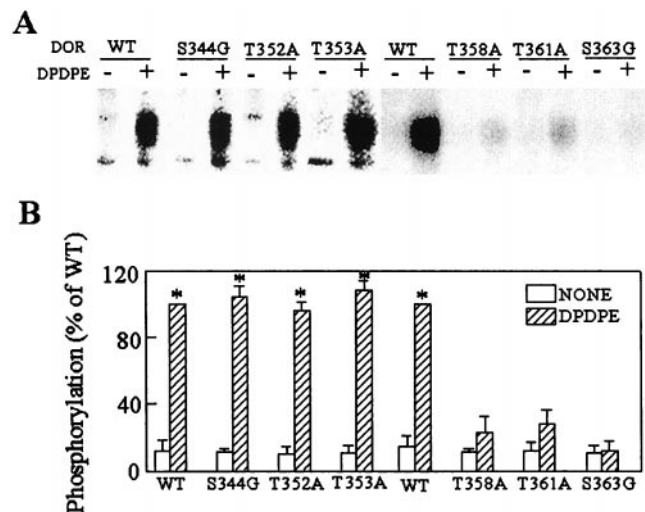

Fig. 4. Agonist-induced phosphorylation of the WT and single point mutant DORs. A, HEK293 cells transiently expressing WT, S344G, T352A, T353A, T358A, T361A, or S363G were metabolically labeled with ³²Pᵢ and stimulated with (+) or without (−) 1 μM DPDPE for 10 min. The DORs were immunoprecipitated with 12CA5, resolved on 10% SDS-polyacrylamide gels, and subjected to phosphorimaging analysis and autoradiography. The pictures are representative of three independent experiments. B, quantitative data of DOR phosphorylation. Data are means ± S.E. of three independent experiments. **$P < .01$ compared with unstimulated control.
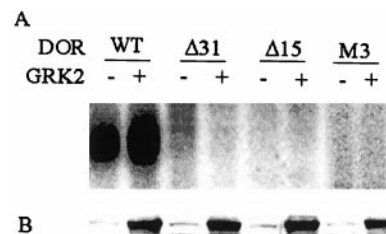

Fig. 3. Agonist-induced phosphorylation of WT, Δ31, Δ15, and M3. HEK293 cells transiently transfected with WT, Δ31, Δ15, or M3 cDNA were metabolically labeled with [³²Pᵢ] and stimulated with (+) or without (−) 1 μM DPDPE for 10 min. The DORs were immunoprecipitated with 12CA5, resolved on 10% SDS-polyacrylamide gels, and subjected to phosphorimaging analysis and autoradiography. The data are representative of three independent experiments.

Fig. 5. Effects of GRK2 overexpression on agonist-induced phosphorylation of WT, Δ31, Δ15, and M3. A, HEK293 cells transfected with WT, Δ31, Δ15, or M3 cDNA alone or cotransfected with GRK2 cDNA were metabolically labeled with ³²Pᵢ and stimulated with 1 μM DPDPE for 10 min. DORs were then immunoprecipitated with 12CA5, resolved on 10% SDS-polyacrylamide gels, and subjected to phosphorimaging analysis and autoradiography. The pictures are representative of three independent experiments. B, in parallel experiments, exogenous expression of GRK2 were analyzed by Western blotting with GRK2 antibodies.

interaction. Analysis of the amino acid sequences flanking T358, T361, and S363 indicates that T358 and S363 reside in putative GRK phosphorylation sites (Onorato et al., 1991), hinting that T361 may be required for GRK recognition. In an attempt to identify which residue is involved in the interaction with GRK2 during agonist stimulation, the three residues were substituted with Asp residues (T358D, T361D, and S363D) to avoid any possible disturbance that Ala or Gly substitution may cause on receptor-GRK interaction. As shown in Fig. 6, phosphorylation of T358D and S363D was not detectable in the cells expressing the receptor alone, a finding similar to that observed with T358A and S363G. However, significant phosphorylation of T361D was detected in response to DPDPE treatment, and the extent of T361D phosphorylation was not significantly different from that of WT (Fig. 6). Furthermore, as observed with WT, overexpression of GRK2 resulted in an 200% increase of T361D phosphorylation, but the T358 and S363 double substitution mutant (D2) was unable to be phosphorylated under the same conditions (Fig. 7). These data indicate that T361 is not a likely primary GRK phosphorylation residue but is a site important for the interaction of the receptor and GRK. The data also support the prediction that T358 and/or S363 serve as GRK phosphorylation sites in a co-operative manner. The capability of T358 and S363 residues to serve as a GRK phosphorylation site was investigated next. Interestingly, phosphorylation of T358D and S363D in response to DPDPE became evident after overexpression of GRK2 (Fig. 7). As show in Fig. 7B, in the presence of overexpressed GRK2, phosphorylation levels of T358D and S363D were approximately half of that of WT and were equivalent to WT phosphorylation determined in the absence of exogenous GRK. These results indicate that both T358 and S363 residues at

the DOR carboxyl terminus could serve as phosphoacceptors of GRK in vivo and opioid-stimulated DOR phosphorylation occurs exclusively at the two GRK sites. However, at physiological concentrations of GRK2, agonist-induced DOR phosphorylation may occur primarily at one site, either T358 or S363.

Discussion

Phosphorylation of opioid receptors is one of the most important steps in the initiation of receptor desensitization, and studies suggest that protein kinases including PKC, GRK, mitogen-activated protein kinase, CaMK, PKA, and protein tyrosine kinases participate in this process (Chen and Yu, 1994; Mestek et al., 1995; Pei et al., 1995; Cai et al., 1996; Polakiewicz et al., 1998; Pak et al., 1999). Overexpression of GRK increases opioid-induced receptor phosphorylation and overexpression of a dominant negative mutant of GRK2 inhibits DOR phosphorylation (Pei et al., 1995), suggesting that GRKs play an important role in agonist-induced opioid receptor phosphorylation. In the present study, we have demonstrated that agonist-stimulated DOR phosphorylation occurred at T358 and S363 residues very close to the receptor cytoplasmic terminus and identified S363 and T358 residues as the sites of phosphorylation by GRK2 in vivo in response to agonist stimulation. These results represent the identification of GRK phosphorylation sites on an opioid receptor for the first time and demonstrate that GRK is the prominent
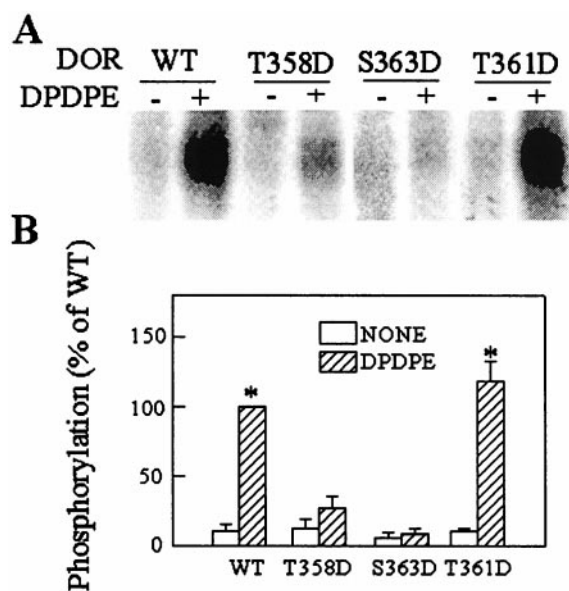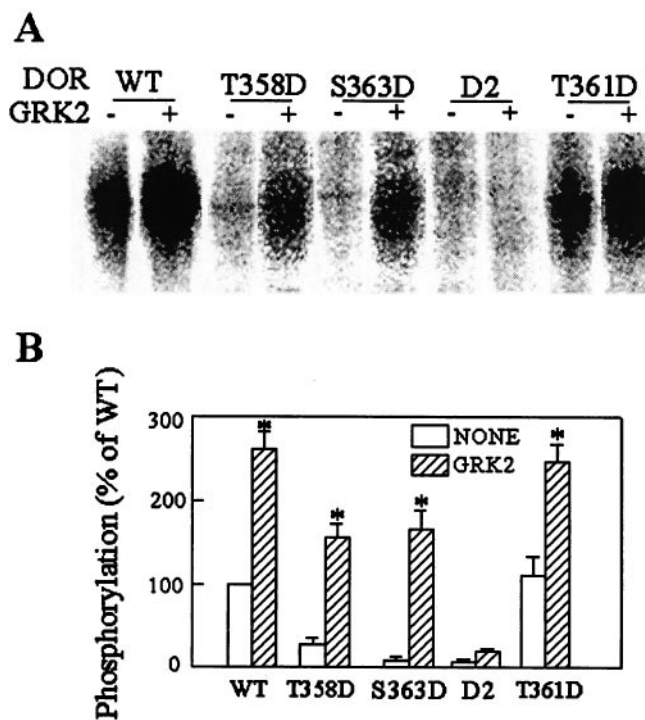

Fig. 7. Effects of GRK2 overexpression on agonist-induced phosphorylation of WT, T358D, T361D, S363D, and D2. A, HEK293 cells transfected with WT, T358D, T361D, S363D, or D2 cDNA alone or cotransfected with GRK2 cDNA were metabolically labeled with $^{32}P_i$ and stimulated with 1 μM DPDPE for 10 min. DORs were immunoprecipitated with 12CA5, resolved on 10% SDS-polyacrylamide gels, and subjected to phosphorimaging analysis and autoradiography. The data are representative of three independent experiments. B, quantitative data of DOR phosphorylation. Data are means ± S.E. of three independent experiments. **P < .01 compared with unstimulated control.

Fig. 6. Agonist-induced phosphorylation of WT, T358D, T361D, and S363D. A, HEK293 cells transiently expressing WT, T358D, T361D, or S363D were labeled with $^{32}P_i$ and stimulated with (+) or without (−) 1 μM DPDPE for 10 min. The receptors were immunoprecipitated with 12CA5, resolved on 10% SDS-polyacrylamide gels, and subjected to phosphorimaging analysis and autoradiography. The picture is representative of three independent experiments. B, quantitative data of DOR phosphorylation. Data are means ± S.E. of three independent experiments. **P < .01 compared with unstimulated control.

kinase responsible for agonist-induced opioid receptor phosphorylation in vivo.

In the presence of exogenously expressed GRK2, significant phosphorylation of T358D and S363D mutants were observed and quantitative analysis of phosphorylation levels revealed that the extents of T358D or S363D phosphorylation reached half of that of WT, whereas significant phosphorylation of D2, the T358 and S363 double mutant, remained undetectable (Fig. 7). This result indicates that both T358 and S363 are phosphorylation sites of GRK, as predicted. In the absence of exogenous GRK, substitutions of either T358 or S363 to an Ala or Gly resulted in almost complete loss of receptor phosphorylation (Fig. 4), suggesting that, in addition to T361, T358 and S363 also take part in the GRK2-receptor interaction, and the integrity of one phosphorylation site is critical for phosphorylation of the other site by GRK. E355 and D364, the two charged residues adjacent to T358 and S363 may also be involved in the receptor-GRK2 interaction and their role remains to be investigated. Substitution of S358 or S363 with Asp also inhibited the receptor-GRK2 interaction but with a less negative effect on the interaction of receptor-GRK2 compared with Ala substitutions. The impairment of receptor phosphorylation caused by substitution of T358 or S363 with Asp residue, mimicking the phosphorylation state suggests that the extra negative charges brought by phosphorylation of one GRK site would have an adverse effect on phosphorylation of the other site by GRK. In other words, phosphorylation of a receptor at one site by GRK would significantly reduce the interaction between receptor and GRK. However, the inhibitory effect of bringing in negatively charged Asp to replace one phosphorylation site on GRK recognition could be overcome by increasing the cellular concentration of GRKs, as demonstrated by overexpression of GRK2 20-fold over the physiological concentration (Fig. 7). These results indicate that, under physiological conditions, agonist-induced DOR phosphorylation occurs primarily at either one of the two GRK sites. These results also suggest that GRK-mediated receptor phosphorylation does not necessarily occur at a precise site in vivo, and it could happen among several sites posing similar structural features preferred by GRK interaction and catalysis. But under physiological conditions, which one of the two GRK sites on a particular receptor gets phosphorylated by GRK upon agonist stimulation may depend on its accessibility and interaction with the enzyme relative to the other site, and it allows that regulation of receptor responsiveness by different GRKs and via more than one mechanism. In the case of DOR, phosphorylation of one residue is generally sufficient for regulation of receptor signaling, and it prevents the receptor from getting hyperphosphorylated.

The essential role of Ser/Thr residues serving as a kinase phosphorylation site has been investigated extensively in phosphorylation of rhodopsin, *N*-formyl peptide receptor, beta-2 adrenergic receptor, C5a anaphylatoxin receptor, and alpha-1B adrenergic receptor (Ohguro et al., 1993; Giannini et al., 1995; Prossnitz et al., 1995; Fredericks et al., 1996; Diviani et al., 1997). However, the participation of the non-phosphate acceptor Ser/Thr residues to receptor phosphorylation has not been addressed. Our data show that T361 flanked by phosphorylation sites T358 and S363 in DOR is critical for the receptor-GRK interaction and clearly demonstrated that Ser and Thr residues could contribute to GRK-mediated phosphorylation as a part of structures required for efficient receptor-GRK2 interaction. In some cases, in addition to acting as a site for GRK phosphorylation, the same Ser or Thr residue could serve as a part of the structure critical for GRK recognition and phosphorylation on another residue. Both of the two functions of Ser/Thr residues are critical for GRK-mediated receptor phosphorylation.

Opioid receptors possess relatively short cytoplasmic termini (50 residues in average) containing a large number of potential Ser/Thr phosphorylation sites (Appleyard et al., 1999). Indirect evidence has hinted that the agonist-induced GRK phosphorylation site(s) is located in the Ser/Thr residues in the receptor cytoplasmic tail. Pak et al. (1997) demonstrated that T394, the most carboxyl-terminal Ser/Thr in MOR, is the primary residue required for agonist-induced MOR desensitization, and they proposed that Thr394 is the primary phosphorylation site of MOR. Recently, Deng et al. (2000) reported that T394 played a crucial role in DAMGO-induced MOR phosphorylation. Appleyard et al. (1999) found that the most carboxyl-terminal potential phosphorylation site, Ser369, is essential for agonist-induced desensitization of rat KOR, implicating the Ser residue closest to the carboxyl terminus as the agonist-induced phosphorylation site of KOR. In the current study, we identified the phosphorylation site in DOR with the $^{32}P_i$-metabolic labeling method. Our research demonstrated that, of the six potential Ser/Thr phosphorylation residues contained in the cytoplasmic tail of mouse DOR, T358, T361, and S363 residues, the three potential phosphorylation sites closest to the receptor carboxyl terminus, are the sites of GRK phosphorylation and/or recognition. These data show that phosphorylation or putative phosphorylation sites of opioid receptors are Ser/Thr residues closest to the carboxyl terminus (at least 30 residues carboxyl terminal from the seventh transmembrane domain). It is likely that the Ser/Thr residues distal from the carboxyl terminus are in closer proximity to the cell membrane, which will restrict their access to and efficient interaction with GRK. In addition, the very carboxyl-terminal portion of the receptor is more flexible in structure and therefore is more accessible to GRK.

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