Hierarchical Phosphorylation of δ-Opioid Receptor Regulates Agonist-induced Receptor Desensitization and Internalization

Odile Maestri-El Kouhen,‡ Guilin Wang, Jonathan Solberg, Laurie J. Erickson, Ping-Yee Law, and Horace H. Loh

From the Department of Pharmacology, Medical School, University of Minnesota, Minneapolis, Minnesota 55455-0217

Treatmen of HEK293 cells expressing the δ-opioid recep\text{-}tor with agonist \text{[D-Pen}^{2,5}\text{]}\text{enkephalin (DPDPE)} resulted in the rapid phosphorylation of the receptor. We constructed several mutants of the potential phosphorylation sites (Ser/Thr) at the carboxyl tail of the receptor in order to delineate the receptor phosphorylation sites and the agonist-induced desensitization and internalization. The Ser and Thr were substituted to alanine, and the corresponding mutants were transiently and stably expressed in HEK293 cells. We found that only two residues, i.e. Thr^{358} and Ser^{363}, were phosphorylated, with Ser^{363} being critical for the DPDPE-induced phosphorylation of the receptor. Furthermore, using alanine and aspartic acid substitutions, we found that the phosphorylation of the receptor is hierarchical, with Ser^{363} as the primary phosphorylation site. Here, we demonstrated that DPDPE-induced rapid receptor desensitization, as measured by adenyl cyclase activity, and receptor internalization are intimately related to phosphorylation of Thr^{358} and Ser^{363}, with Thr^{358} being involved in the receptor internalization.

Prolonged exposure to opioid drugs often produces tolerance, dependence, and addiction, and the molecular mechanisms underlying these phenomena are still poorly understood. Opioid receptors belong to the G protein-coupled receptor (GPCR) superfamily (1, 2). Therefore, one component of opioid tolerance is likely to be mediated by a phosphorylation-dependent desensitization of the receptor. Phosphorylation of the prototypic \text{β}_{2}-adrenergic receptor by protein kinases, including the G protein-coupled receptor kinases (GRKs), promotes the association with the receptor of inhibitory proteins called arrestins (3, 4). This association uncouples the receptor from the G proteins and promotes targeting of activated receptors to clathrin-coated pits for subsequent internalization, thus blunting receptor signaling.

Agonist-induced phosphorylation of opioid receptors has been demonstrated in different systems (5–10). Concrete demonstration of the phosphorylation of δ-opioid receptor (DOR) was first reported by Pei and colleagues (6). Although the sites of agonist-dependent phosphorylation have not yet been identified, truncation of the carboxyl tail (C-tail) of DOR showed that major phosphorylation sites are localized within this domain (11, 12). Phosphorylation of DOR appears to be the mechanism for agonist-induced receptor desensitization (6, 13, 14). \text{[D-Pen}^{2,5}\text{]}\text{enkephalin (DPDPE)}-induced phosphorylation of DOR seems to involve one or more GRKs (6), and Ala substitution of the last four carboxyl-terminal Ser and Thr of the receptor impaired the GRK- and arrestin-mediated receptor desensitization (13). In \text{Xenopus} oocytes, co-expression of GRK3 and β-arrestin resulted in an increased rate of agonist-induced homologous desensitization of DOR (13). However, truncation of the COOH-terminal 31 amino acids of DOR did not affect the agonist-induced desensitization of the receptor in CHO cells (15), unlike what Zhao and colleagues (11) reported in NG108-15 cells. Opioid receptors are endocytosed in a dynamin-dependent manner by clathrin-coated pits (14, 16–18). However, the precise role of phosphorylation in the mechanism of opioid receptor endocytosis is still not fully understood. A truncated mutant δ-opioid receptor undergoes rapid agonist-induced internalization in HEK293 cells, but is not phosphorylated in the presence of agonist, whereas the same mutant remained predominantly in the plasma membrane of CHO cells, suggesting that cell type-specific differences may exist in the biochemical requirements for the agonist-induced endocytosis (12).

In light of these discrepancies, it is imperative to identify the agonist-induced phosphorylation sites and to investigate the role of the phosphorylation of these sites in the regulation of the δ-opioid receptor. We used series of receptor mutants to identify the phosphorylation sites at the C-tail of DOR. In this study, we reported that two sites (Thr^{358} and Ser^{363}) are phosphorylated in the presence of DPDPE and, furthermore, that the phosphorylation of the receptor is hierarchical. Additionally, we investigated the role played by these two phosphorylation sites in internalization of the receptor. Here, we also demonstrated that internalization of the activated receptor plays a role in the loss of δ-opioid receptor-mediated inhibition of adenyl cyclase activity.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium and Genetin (G418) were purchased from Life Technologies, Inc. [\text{3H}]\text{Diprenorphine (58 Ci/mmol)} was supplied by Amersham Pharmacia Biotech and [\text{32P}]Orthophosphate (>400 Ci/mmol) by ICN (Costa Mesa, CA). [\text{32P}]Acetylated cAMP (2200 Ci/mmol) was purchased from Linco Research Inc. (St. Charles, MO). Polyclonal antibodies that recognize the acetylated cAMP were purchased from Calbiochem (La Jolla, CA). NIDA (National Institutes on Health, Bethesda, MD) supplied the \text{[D-Pen}^{2,5}\text{]}\text{enkephalin ligand. All other chemicals were purchased from Sigma.}

Generation of the Mutants of the δ-Opioid Receptor—The human influenza virus hemagglutinin (HA) epitope-tagged mouse δ-opioid re-
to generate most of the point mutations. Ser and/or Thr present at the C-tail of the receptor (from Thr<sup>335</sup> to Ser<sup>363</sup>) were point-mutated to Ala or Asp by oligonucleotide-directed mutagenesis using a QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, CA) according to the manufacturer’s directions, except for the following mutants. The T358A, S363A, and S363D mutants were constructed using the Altered Sites<sup>™</sup> in vitro mutagenesis system provided by Promega Corp. (Madison, WI), using DOR-1 cDNA subcloned into the phagemid pAlter-1 as template. The nucleotide sequences of all mutants were confirmed by direct DNA sequencing using Sequenase II. The EcoRIV-XbaI fragments of the different plasmids were excised and ligated to the Dor tag in pcDNA3 with the same fragment removed.

Cell Culture and Transfections—Human embryonic kidney HEK293 cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 IU/ml penicillin under humidified atmosphere at 5% CO<sub>2</sub>. Cells were transiently transfected by the calcium phosphate precipitation method, and the assays were performed 48 h after transfection. Pool of stably transfected cells expressing the wild-type or mutant receptors were isolated in the presence of 1 mg/ml G418, without selection of individual clones, to avoid any position effect due to the random integration of each cDNA into the chromosomes. Receptor expressions were determined by whole cell binding experiments ([3H]Diprenorphine in 25 mM HEPES buffer, pH 7.6).

Specific binding is defined as the difference between the radioactivity bound to the cells in the absence and presence of 100 μM naloxone. HEK293 cells stably expressing the ecysosine receptor in the pVXrXr vector (Ecr9-23 cells) were purchased from Invitrogen and clones stably co-expressing the wild-type or S363A mutant receptors were described in Ref. 21. 0.2 μM or 2 μM ponasterone A (PA), used to induce respectively low (0.37 ± 0.14 pmol/mg of protein for the wild-type receptor; 0.14 ± 0.1 pmol/mg of protein for the S363A mutant) or high (1.0 ± 0.5 pmol/mg of protein for the wild-type receptor; 1.15 ± 0.7 pmol/mg of protein for the S363A mutant) receptor level, were added 48 h before assays.

Opioid Inhibition of Intracellular cAMP Level—HEK293 cells seeded in 100-mm dishes were transiently transfected with 10 μg of HA epitope-tagged DOR cDNA or empty vector (mock) were labeled with <sup>32</sup>P, and stimulated with 1 μM DPDPE for 30 min. The receptors were purified as described under “Experimental Procedures” and analyzed by phosphorimaging (a) and immunoblotting (b).

FIG. 1. In vivo DPDPE-induced phosphorylation of wild-type δ-opioid receptor. HEK293 cells transiently transfected with 10 μg of HA epitope-tagged DOR cDNA or empty vector (mock) were labeled with <sup>32</sup>P, and stimulated with 1 μM DPDPE for 30 min. The receptors were purified as described under “Experimental Procedures” and analyzed by phosphorimaging (a) and immunoblotting (b).

Fluorescence flow cytometry (FACScan, Becton Dickinson, Palo Alto, CA).

Data Analysis—Data were analyzed using the GraphPad program. Mean values from individual treatment groups were statistically analyzed by a one-way analysis of variance with subsequent comparisons among treatment groups from their control by Student’s t test.

RESULTS

To investigate the in vivo phosphorylation properties of DOR, HEK293 cells expressing the wild-type receptor were radiolabeled with [32P]orthophosphate (<sup>32</sup>P), then treated with a saturating concentration (1 μM) of DPDPE, and finally receptors were immunopurified and analyzed by SDS-PAGE autoradiography (Fig. 1a). Non-specific signal in these experiments was negligible (lane 1). In presence of DPDPE, phosphorylation of the receptor was strongly stimulated (lane 3) when compared with the basal phosphorylation in absence of agonist (lane 2) and revealed a diffuse phosphoprotein band migrating at approximately 50–60 kDa, corresponding to that of the HA epitope-tagged DOR (Fig. 1b). Phosphorylation of this protein band was time- and concentration-dependent (data not shown), and the phosphorylation patterns were similar in HEK293 cells either transiently or stably expressing the receptor (Figs. 1, 3, and 4). Additional immuno-reactive species were observed in some experiments with an apparent molecular mass corresponding to the receptor dimers, consistently with previously described oligomers of DOR (19, 20).

Quantitation of δ-Opioid Receptor Internalization by Fluorescence Flow Cytometry—Stably transfected HEK293 cells expressing wild-type or mutant HA epitope-tagged receptors were treated with 1 μM DPDPE for the indicated times. Cells were chilled on ice to stop membrane trafficking, and receptors were visualized by using a 1:400 dilution of the high affinity mouse monoclonal anti-HA antibody (HA.11 clone 16B12; Babco, Richmond, CA) and a 1:500 dilution of the secondary antibodies goat anti-mouse IgG conjugated with Alexa488 (Molecular Probes, Eugene, OR). Incubations were performed at 4 °C. Surface receptor staining intensity of antibody-labeled cells was analyzed using fluorescence flow cytometry (FACScan, Becton Dickenson, Palo Alto, CA).

Data Analysis—Data were analyzed using the GraphPad program. Mean values from individual treatment groups were statistically analyzed by a one-way analysis of variance with subsequent comparisons among treatment groups from their control by Student’s t test.
Phosphorylation of DOR Is Hierarchical

T355A, S344A, T352A, or T353A mutants did not display any significant differences in agonist-induced receptor phosphorylation, indicating that none of these four residues are involved in the DPDPE-induced phosphorylation of the receptor (Fig. 3, lanes 3–6). In contrast, mutation of Thr358, Thr361, or Ser363 to Ala dramatically reduced the phosphorylation level to about 47.7%, 66.3%, or 91.8%, respectively, as compared with the wild-type receptor, indicating that these residues are involved in the agonist-induced phosphorylation of the receptor (Fig. 3). Interestingly, little or no agonist-induced phosphorylation could be detected with the S363A mutant, indicating that Ser363 is the primary phosphorylation site. The complete blockade of the DPDPE-induced phosphorylation of the receptor by the S363A mutation (Figs. 3 and 4), suggested that this residue is either a phosphorylation site or is a kinase recognition site. The actual phosphorylation of this site was demonstrated by the mutant receptor in which all the Ser/Thr residues at the C-tail, except Ser363, were substituted to Ala (mutant CT/361T/S363D, Fig. 4). This mutant showed a significant agonist-induced phosphorylation and reached 36.6 ± 3.2% (n = 9) of that of the wild-type receptor. This result showed clearly that Ser363 is the primary phosphorylation site, and that phosphorylation of DOR could be hierarchical with Ser363 being the first residue to be phosphorylated.

In order to identify the other phosphorylation sites, Ser363 was mutated to aspartic acid (Fig. 4), which mimics a phosphorylated form of this residue, and which should induce a recovery of the receptor phosphorylation, if the mechanism is hierarchical. As shown in Fig. 4, the S363D mutant restores the DPDPE-induced phosphorylation (compared with the S363A mutant) to about 27.5% of that of the wild-type receptor. This result confirmed, therefore, that Ser363 has to be phosphorylated before another site, and that Thr358 and/or Thr361 is (are) the other phosphorylation site(s). No phosphorylation, however, could be detected with a mutant leaving only Thr358 as a potential phosphorylation site along with the Asp-substituted Ser363 (mutant CT/361T/S363D), suggesting, therefore, that Thr356 is not phosphorylated right after Ser363 but rather Thr358 is. Indeed, the extent of phosphorylation in the T358A mutant is not significantly different (p > 0.1) from the extent of phosphorylation in the mutant leaving Ser363 as the only potential phosphorylation site (mutant CT/363S). Consistently, the lower level of phosphorylation in the S363D mutant (reaching only about 27.5% of that of the wild-type receptor) confirms that only one site is phosphorylated along with Ser363 and that this other residue is Thr358. Nevertheless, the T361A mutation...
affected the agonist-induced phosphorylation of the receptor. Thr\textsuperscript{361} could participate in the phosphorylation of the receptor as a kinase recognition/binding site but not as a phosphorylation site. The T361D mutant, which was originally constructed to define the phosphorylation mechanism of DOR, exhibiting almost but not complete recovery of the phosphorylation level as compared with that of the wild-type receptor, might affect to almost but not complete recovery of the phosphorylation level to define the phosphorylation mechanism of DOR, exhibiting as a kinase recognition/binding site but not as a phosphorylation site. This observed recovery was, however, blocked when Ser363 was mutated into Ala along with the T361D substitution (mutant T361D/S363A, Fig. 3), consistently with the critical role of Ser363 in the phosphorylation of the receptor. 

Next, we examined the role of phosphorylation in the agonist-induced desensitization of DOR in HEK293 cells. The kinetics of the loss of DPDPE inhibition of the adenyl cyclase activity were monophasic, and the apparent rate of desensitization was relatively slow, with a $t_{1/2}$ of about 1.3 ± 0.25 h (n = 6, data not shown), consistently with previous study (10). In contrast, DPDPE-induced phosphorylation of the receptor followed much faster kinetics, showing a maximum within 10 min of DPDPE exposure (data not shown). The difference in these two rates suggests that receptor phosphorylation does not directly lead to DOR desensitization. The failure to correlate phosphorylation to desensitization of DOR could be due to the relatively high level of receptor expression (2.2 ± 0.8 pmol/mg of protein) in HEK293 cells. In an earlier study, by controlling the expression level of the receptor in HEK293 cells with an inducible-expression system to control the expression of DOR, HEK293 cells stably expressing the wild type or mutant receptors were labeled, and the ability of 1 μM DPDPE to induce phosphorylation was determined as described under “Experimental Procedures.” Top, amino acid sequence of the intracellular C-tail of DOR. The different Ala or Asp substitutions are indicated, and the dashed lines represent no changes from the wild-type sequence. Bottom, receptors were purified and resolved on a SDS-PAGE. After quantification by phosphorimaging and immunoblotting, intensities of the phosphorylated bands were expressed as a percentage of DPDPE-induced phosphorylation of the wild-type receptor. Data shown represent mean ± S.E. of at least three separate experiments. ***, p < 0.001 compared with the phosphorylation pattern of the S363A mutant.

Since internalization is involved in the desensitization of several GPCRs (4), and since in our earlier study (21) we demonstrated that receptor internalization also participated in the rapid desensitization of DOR, we examined whether the DPDPE-induced phosphorylation of Thr\textsuperscript{361} and Ser\textsuperscript{363} would lead to receptor internalization. The extent of internalization of the wild type and mutant receptors from the cell surface were examined using flow cytometry (Fig. 6). Kinetics analysis indicated that DOR internalized rapidly in presence of DPDPE and that about 80% of total cell surface receptor were internalized in response to 1 h agonist treatment, with a $t_{1/2}$ = 10.8 ± 1.6 min (Table II). This rate compared favorably with previous reports (12, 17, 21). Substitution of the four first Ser or Thr at the C-tail into Ala (T335A to T353A, Fig. 3), consistently with the critical role of Ser\textsuperscript{363} in the phosphorylation of DOR at Ser\textsuperscript{363} is an important but not obligatory event in the DPDPE-induced desensitization.
Phosphorylation of DOR Is Hierarchical

These results suggest that phosphorylation of Thr$^{358}$ is critical for receptor internalization. Indeed, the S363D mutant (Table II, group I), allowing the DPDPE-induced phosphorylation of Thr$^{358}$, exhibited a pattern of receptor internalization identical to the wild-type receptor. Therefore, internalization of DOR required the phosphorylation of Thr$^{358}$, subsequent to the phosphorylation of Ser$^{363}$.

**DISCUSSION**

From our current mutagenesis studies, it is apparent that Ser$^{363}$ is the primary phosphorylation site, that agonist-induced phosphorylation of DOR occurs in a hierarchical manner, and that Thr$^{358}$ and Ser$^{363}$ are the only two residues being phosphorylated. Thr$^{361}$ participates in receptor phosphorylation, since its substitution into Ala reduced the extent of phosphorylation when compared with the wild-type receptor. Probably, the remaining phosphorylation observed with the T361A mutant comes from Ser$^{363}$, since Ser$^{363}$ is phosphorylated independently of Ser/Thr present at the C-tail of the receptor (mutant CT/363S, Fig. 4). Mutation of Thr$^{361}$ could impair the recognition/binding of a kinase to phosphorylate Thr$^{358}$, thereby reducing the receptor phosphorylation level. Hierarchical phosphorylation has been demonstrated for only a few GPCRs, including the rhodopsin and the N-formyl peptide receptor (22, 23). Whether this phosphorylation mechanism is conserved among members of the GPCR family, or specific to some of them, remains to be demonstrated. It is possible that phosphorylation of Ser$^{363}$ creates a new acidic phosphoserine recognition sequence for the subsequent phosphorylation of Thr$^{358}$ by the same or by another kinase. Several groups have proposed that members of the GRK family can phosphorylate DOR (6, 24). Although the consensus motif of GRK among various GPCRs has not been clearly defined, GRK normally phosphorylate Ser/Thr residues adjacent to an acidic or charged amino acid residue (25). Interestingly, Ser$^{363}$ and Thr$^{358}$ are immediately downstream of Pro and Val residues, respectively (Fig. 2). Ser$^{363}$ is upstream from an aspartic residue, which could serve as a GRK recognition motif. However, Thr$^{358}$ is upstream from an Ala residue. Whether phosphorylation of Ser$^{363}$ and Thr$^{358}$ involved a GRK remains to be demonstrated. Some reports suggested that agonist-induced phosphorylation of the opioid receptors could be mediated by Ca$^{2+}$/calmodulin-dependent protein kinase II (26), or by mitogen-activated protein kinase (27), but the amino acid motif surrounding Thr$^{358}$ or Ser$^{363}$ does not correspond to that of either kinase. Thus, it is tempting to suggest that a yet unknown Ser/Thr kinase is responsible for the DPDPE-induced phosphorylation of DOR. The involvement of this unidentified kinase might be one of the reasons why overexpression of a dominant negative mutant of GRK could not completely block the agonist-induced phosphorylation of the receptor (6, 10).

The rapid and slow desensitization of DOR is dependent on the relative level of expression of the receptor. The slow rate of a loss of DPDPE inhibition of forskolin-stimulated adenyl cyclase in cells expressing a relatively high level of receptors (Fig. 5) does not correlate with the rapid DPDPE-induced phosphorylation of the receptor. The relatively high level of receptor expressed at the cell surface that is not phosphorylated and sufficiently high enough to maintain the agonist-mediated activity along with the high efficient coupling between DOR and the adenyl cyclase (28), could explain why the phosphorylation of the receptor did not correlate with the loss of response. The fact that the mutants showing no detectable agonist-induced phosphorylation, such as T361A/S363A and S363A mutants (data not shown and Fig. 5), which exhibit a similar rate and extent of desensitization as the wild-type receptor at relatively high receptor levels, suggests

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**TABLE II**

| Group     | Del                       | $t_{1/2}$ (min) | Internalization (%) |
|-----------|----------------------------|----------------|---------------------|
| Group I   |                           |                |                     |
| T335A     |                           | 11.4 ± 0.2     | 80.6 ± 2.4          |
| T335A     |                           | 7.6 ± 1.4      | 78.2 ± 4.5          |
| T352A     |                           | 15.2 ± 1.3     | 76.8 ± 9.5          |
| T353A     |                           | 14.2 ± 2.6     | 70.8 ± 2.7          |
| S363D     |                           | 12.2 ± 0.6     | 74.6 ± 2.2          |
| DTS       |                           | 17.3 ± 0.9     | 63.3 ± 11.5**       |
| CT/363S   |                           | 17.1 ± 3.1     | 56.4 ± 1.8**        |
| CT/A363S  |                           | 12.3 ± 2.4     | 60.8 ± 2.6**        |

DPDPE-induced internalization of the receptor in HEK293 cells (Table II, group I). In contrast, the DTS mutant as well as the S363A mutant, both blocking the DPDPE-induced phosphorylation of the receptor, revealed a substantially slower rate and lower extent of internalization, strongly suggesting that phosphorylation of Ser$^{363}$ and/or Thr$^{358}$ contributed to the receptor internalization (Fig. 6 and Table II). Phosphorylation of Ser$^{363}$ alone (mutant CT/363S) prevented the receptor from internalizing as efficiently as the wild-type receptor, suggesting that the phosphorylation of this Ser only is insufficient to induce receptor internalization. Ala substitution of Thr$^{358}$ also blunted the agonist-induced internalization of the receptor.
that events other than receptor phosphorylation e.g. internalization/sequestration/down-regulation could be involved in the slow desensitization of the receptor. At low levels of receptor expression, similar to the levels of expression observed in endogenously expressing cells, the blockade of the agonist-induced receptor phosphorylation dramatically attenuated the magnitude of rapid desensitization, implying that the receptor phosphorylation at Ser363 participates in receptor regulation. Nevertheless, the ability of the receptor to still desensitize indicates that the agonist-induced internalization contributes also to the loss of response, as it was shown for other GPCRs, like for example for the m3 mAch receptor (29) or the µ-opioid receptor (30, 31). We recently reported that, at low expression levels of µ- and δ-opioid receptors, a blockade of receptor internalization leads to a blockade of the agonist-induced rapid desensitization of these receptors (21, 30).

Mutation of Thr358 attenuates the agonist-induced internalization of DOR, implying that phosphorylation of this residue is crucial for receptor internalization and hence contributed to the DPDPE-induced rapid desensitization. Down-regulation of DOR (32, 33), which involves receptor sequestration and degradation, likely contributes to the slow loss of receptor activity, regardless of whether or not the receptor is phosphorylated (Fig. 5). Thr358 has been reported to be required for the agonist-induced down-regulation of DOR in CHO cells (34). Since the DPDPE-induced phosphorylation of DOR in HEK293 cells does not involve Thr358 (Fig. 3), the ability of the receptor to down-regulate will not be affected by the inability of the receptor to be phosphorylated. Similarly, for the µ-opioid receptor, mutation of Ser363 to Ala attenuated agonist-induced down-regulation without being an agonist-induced phosphorylation site (35), showing that phosphorylation of this particular site is not necessarily a signal for the processing of receptor traffic at the early endosome. Whether or not Thr358 is involved in DPDPE-induced down-regulation of the receptor in HEK293 cells remains to be demonstrated, since a truncated δ-opioid receptor remaining predominantly in the plasma membrane of CHO cells, can still internalize in HEK293 cells (12). Alternatively, a NPXXY motif, common to many GPCRs including the opioid receptors (36), present at the interface between the seventh transmembrane and the C-tail of the receptor, or a di-Leu-based motif (37) present within the third cytoplasmic domain of DOR, could be implicated in the trafficking of the receptor.

We can propose that phosphorylation of Ser363 will promote the uncoupling of the activated receptor from its cognate G protein. Then, phosphorylation of Thr358 will regulate internalization of the receptor, further attenuating receptor-mediated signaling. Therefore, functional uncoupling of receptor and G protein, followed by endocytosis, will blunt the response to agonists. Native opioid peptides or opiate drugs are relatively resistant to proteolytic degradation in the extracellular environment. Therefore, opioid receptors may use this regulation mechanism to alter persistent activation. Whether the same mechanism persists in all in vitro cell models or in vivo remains to be demonstrated.

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