

The N-terminal Extension of $G\alpha_q$ Is Critical for Constraining the Selectivity of Receptor Coupling*

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Evi Kostenis, Michael Y. Degtyarev‡, Bruce R. Conklin‡, and Jürgen Wess§

From the Laboratory of Bioorganic Chemistry, NIDDK, National Institutes of Health, Bethesda, Maryland 20892 and the ‡Departments of Medicine and Pharmacology, Gladstone Institute of Cardiovascular Disease, University of California, San Francisco, California 94141-9100

Characteristically, an individual member of the superfamily of G protein-coupled receptors can interact only with a limited number of the many structurally closely related G protein heterotrimers that are expressed within a cell. Interestingly, the N termini of two G protein α subunits, $G\alpha_q$ and $G\alpha_{11}$, differ from those of other α subunits in that they display a unique, highly conserved six-amino acid extension. To test the hypothesis that this sequence element is critical for proper receptor recognition, we prepared a $G\alpha_q$ deletion mutant ($-6q$) lacking these first six amino acids. The $-6q$ construct (or wild type $G\alpha_q$ as a control) was coexpressed (in COS-7 cells) with several different $G_{i/o}$ - or G_s -coupled receptors, and ligand-induced increases in inositol phosphate production were determined as a measure of G protein activation. Whereas these receptors did not efficiently interact with wild type $G\alpha_q$, most of them gained the ability to productively couple to $-6q$. Additional experiments indicated that the observed functional promiscuity of $-6q$ is not due to overexpression (as compared with wild type $G\alpha_q$) or to a lack of palmitoylation. We conclude that the N-terminal extension characteristic for $G\alpha_{q/11}$ proteins is critical for constraining the receptor coupling selectivity of these subunits, indicative of a novel mechanism by which the fidelity of receptor-G protein interactions can be regulated.

G protein-coupled receptors (GPCRs),¹ when activated by extracellular ligands, interact with specific classes of heterotrimeric G proteins (consisting of α , β , and γ subunits) which can then, in their activated forms, inhibit or activate various effector enzymes and/or ion channels (1–5). Characteristically, a specific GPCR can interact with only a limited subset of the

many structurally similar G proteins that are expressed within a cell. Molecular genetic and biochemical studies have identified distinct intracellular regions (as well as single amino acids contained within these domains) on the GPCR proteins that play key roles in determining the fidelity of receptor-G protein coupling (1–7). In addition, recent studies have shown that residues at the extreme C terminus of the G protein α subunits are also of fundamental importance for the selectivity of receptor-G protein interactions (8–10). However, several lines of evidence suggest that the C terminus of the $G\alpha$ subunits is clearly not the only structural determinant on the G proteins that is critical for dictating receptor-G protein coupling selectivity (2, 5).

Interestingly, two G protein α subunits, $G\alpha_q$ and $G\alpha_{11}$, contain a unique six-amino acid extension that is not found in other $G\alpha$ subunits (Fig. 1). This short sequence is highly conserved among all vertebrate species from which these subunits have been cloned so far (11–15), suggesting that it may be relevant for some aspect of $G\alpha_q/G\alpha_{11}$ function.

Previous studies (16, 17) analyzing the biochemical properties of a mutant $G\alpha_q$ subunit lacking the N-terminal extension (hereafter referred to as $-6q$) failed to reveal any major functional differences between wild type $G\alpha_q$ (WTq) and $-6q$. Both studies showed that $G_{q/11}$ -coupled receptors such as the NK2 neurokinin (16) and the m1 muscarinic receptor (17) were able to activate $-6q$ in a fashion identical to WTq. Similarly, other functional properties, such as their affinity for $\beta\gamma$ subunits and their ability to activate downstream effectors (e.g. phospholipase C β (PLC β)), were also found to be very similar for the two G protein subunits (16, 17).

In this study, we tested the hypothesis that the N-terminal extension in WTq may play a role in maintaining the selectivity of receptor recognition, an issue that had not been addressed yet. Toward this goal, the ability of several different $G_{i/o}$ - and G_s -coupled receptors to interact with WTq or $-6q$ were examined in cotransfected COS-7 cells. Whereas none of the receptors (upon incubation with the appropriate agonist ligands) was able to activate WTq to a significant extent, most of the receptors gained the ability to couple to $-6q$ with considerable efficiency (measured biochemical response: stimulation of phosphatidylinositol (PI) hydrolysis). These data suggest that the N-terminal extension characteristic for $G\alpha_q$ and $G\alpha_{11}$ subunits is critical for constraining the receptor coupling selectivity of these proteins.

EXPERIMENTAL PROCEDURES

Creation of G Protein Expression Plasmids—To create a construct coding for a mutant $G\alpha_q$ subunit lacking the first six amino acids ($-6q$), a pcDNAI-based expression plasmid coding for murine WTq (11, 18) was used. To generate the $-6q$ expression plasmid, a 78-base pair synthetic *Bam*HI-*Fsp*I fragment containing the desired deletion was used to replace the corresponding sequence in the wild type plasmid. In both plasmids (WTq and $-6q$), the *Bam*HI site of the pcDNAI polylinker was immediately followed by the initiating ATG codon. Both plasmids contained a short sequence coding for an internal hemagglutinin (HA) epitope tag (DVPDYA), which replaced WTq residues 125–130 (18). The presence of the epitope tag did not affect the receptor and effector coupling properties of WTq (8, 9, 18). The identity of the two G protein constructs was verified by dideoxy sequencing (19).

Transient Expression of Receptor and G Protein Constructs—COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37 °C in a humidified 5% CO₂ incubator. For transfections, 1×10^6 cells were seeded into 100-mm dishes. About

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‡ To whom correspondence should be addressed: NIDDK, NIH, Lab. of Bioorganic Chemistry, Bldg. 8A, Rm. B1A-09, Bethesda, MD 20892. Tel.: 301-402-4745; Fax: 301-402-4182.

¹ The abbreviations used are: GPCR, G protein-coupled receptor; HA, hemagglutinin; IP₁, inositol monophosphate; PI, phosphatidylinositol; PLC, phospholipase C; WTq, wild type $G\alpha_q$; $-6q$, wild type $G\alpha_q$ lacking the first six amino acids; PTX, pertussis toxin; PAGE, polyacrylamide gel electrophoresis.

	αN Helix				
	1	7	9	10	
α _q (WTq)	MTLESIMAC	CLS	EEAK	-
-6q		MAC	CLS	EEAK
α ₁₁	MTLESMMAC	CLS	DEVK	-
α _{i1,3}	MGC	TLS	AEDK	-
α _{o1,2}	MGC	TLS	AEER	-
α _{t1}	MGA	GAS	AEEK	-
α _s	MGCLGNSK	TEDQRNEEK			-

FIG. 1. Comparison of the N-terminal amino acid sequences of selected G protein α subunits. Sequences (human) were taken from Refs. 15 and 37 (note that the human α_q sequence (15) shown here is identical to the corresponding mouse sequence (11)). Gaps were introduced to allow for maximum sequence identity. The position of the N-terminal portion of the α N helix, as revealed by x-ray crystallography (41, 42) is indicated. -6q denotes a mutant $G\alpha_q$ construct (mouse) lacking the first six amino acids.

24 h later, COS-7 cells were cotransfected with expression plasmids coding for WTq or -6q (1 μ g DNA/dish) and the indicated receptor cDNAs (4 μ g DNA/dish) by using a DEAE-dextran procedure (20). The following receptor expression plasmids were used: m2 muscarinic receptor in pcD (21), D2 dopamine receptor (22) in pcDNA1, κ -opioid receptor (23) in pcDNA3, somatostatin SSTR1 receptor (24) in pCMV, A1 adenosine receptor (25) in CDM7, D1 dopamine receptor (26) in pcDNA1, V2 vasopressin receptor in pcD-PS (27), and β_2 -adrenergic receptor (28) in pSVL.

PI Hydrolysis Assays—Approximately 24 h after transfections, cells were split into 6-well dishes ($\sim 0.4 \times 10^6$ cells/well) in culture medium supplemented with 3 μ Ci/ml [3 H]myo-inositol (20 Ci/mmol; American Radiolabeled Chemicals Inc.). After a 24-h labeling period, cells were preincubated for 20 min at room temperature with 2 ml of Hanks' balanced salt solution containing 20 mM HEPES and 10 mM LiCl. Cells were then stimulated in the same buffer with the appropriate agonist ligands (1 h at 37 °C), and increases in intracellular inositol monophosphate (IP₁) levels were determined by anion exchange chromatography as described (10).

In a subset of experiments, transfected cells were incubated with pertussis toxin (PTX; 500 ng/ml) for the last 18–24 h of culture.

[3 H]Palmitate Labeling—Approximately 48 h after transfections, COS-7 cells were metabolically labeled for 1 h with 800 μ Ci/ml of [9,10- 3 H]palmitate (60 Ci/mmol; American Radiolabeled Chemicals Inc.) in 5 ml of serum-free medium supplemented with 1% (v/v) dimethyl sulfoxide as described (29). The chemical nature of [3 H]palmitate incorporation into G protein α subunits has been characterized earlier (18, 30) in cells radiolabeled under similar conditions.

Cell Fractionation—Cells were fractionated into particulate and soluble fractions as described (29).

Immunoprecipitation and Immunoblotting—The 12CA5 mouse monoclonal antibody (BAbCo) specific for the HA epitope was used for immunoprecipitation and immunoblotting. Immunoprecipitation studies were performed using equivalent amounts of protein (2.5 mg) from total cell suspension in solubilization buffer consisting of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% (w/v) of Nonidet P-40, 0.5% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS in a total volume of 1 ml. Cell suspensions were added to the solubilization buffer and incubated at 4 °C on a rotator for 30 min followed by centrifugation for 10 min at 14,000 rpm in an Eppendorf 5415 microcentrifuge to pellet insoluble material. Supernatants were transferred to fresh Eppendorf tubes, and 5 μ g of 12CA5 antibody and 20 μ l of a 50% suspension of protein A-agarose (Sigma) were added followed by an overnight incubation at 4 °C on a rotator. Immunoprecipitates were recovered by centrifugation at 1,000 \times g in a microcentrifuge, washed twice in 1 ml of solubilization buffer containing 1/10 of the original detergent concentration, solubilized in 45 μ l of gel sample buffer (Novex) in the absence of reducing agents, boiled for 5 min, separated by SDS-PAGE on 10% Tris-Gly gels (Novex), and prepared for fluorography using Amplify (Amersham Corp.) according to the manufacturer's instructions. Fluorograms were exposed for 4–6 weeks at -70 °C.

Immunoblotting was performed by separating equal amounts of protein (100 μ g) from subcellular fractions solubilized in gel sample buffer (Novex) with 2.5% (v/v) β -mercaptoethanol on 10% Tris-Gly gels (Novex), transfer to nitrocellulose membranes, probing with the 12CA5 antibody conjugated to horseradish peroxidase (Boehringer Mannheim), and development with enhanced chemiluminescence reagents (Amersham Corp.). Protein concentrations were determined using the

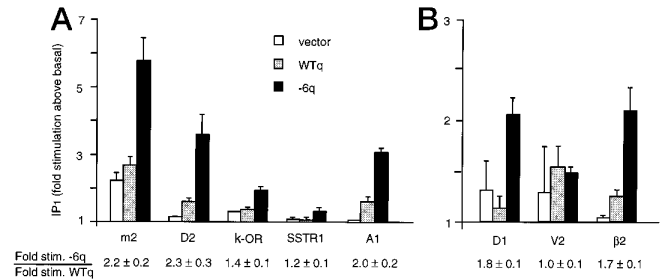


FIG. 2. Functional interaction of different G protein-coupled receptors with the $G\alpha$ subunits, WTq and -6q. COS-7 cells coexpressing WTq or -6q and different $G_{i/o}$ (A) or G_s -coupled receptors (B) were incubated for 1 h (at 37 °C) in the absence or the presence of the indicated agonist ligands. The resulting increases in intracellular IP₁ levels were determined as described under "Experimental Procedures." Data are given as the means \pm S.E. of three to seven independent experiments, each carried out in triplicate. The following ligands were used: for A, m2 muscarinic receptor: carbachol (100 μ M); D2 dopamine receptor: (-)-quinpirole (10 μ M); κ -opioid receptor (κ -OR): (-)-U50488 (10 μ M); somatostatin SSTR1 receptor: somatostatin-14 (1 μ M); for B, A1 adenosine receptor: R(-)-PIA (R(-)-N⁶-(2-phenylisopropyl)-adenosine; 10 μ M), D1 dopamine receptor: dopamine (1 mM); V2 vasopressin receptor: [Arg⁸]vasopressin (1 nM); β_2 -adrenergic receptor: (-)-isoproterenol (200 μ M). Numbers underneath the figures are ratios obtained by dividing the fold PLC stimulation seen with -6q by the corresponding WTq value. These ratios indicate that the relative increase in PLC activation (-6q versus WTq) was similar for the m2, D2, A1, D1, and β_2 receptors.

Bio-Rad protein assay kit with IgG as the standard.

Drugs—[Arg⁸]vasopressin, (-)-isoproterenol, and PTX were purchased from Sigma. All other ligands used in this study were obtained through Research Biochemicals Inc.

RESULTS AND DISCUSSION

Receptor Coupling Properties of WTq and -6q—Initially, a series of receptors that are preferentially coupled to G proteins of the $G_{i/o}$ family (m2 muscarinic, D2 dopamine, κ -opioid, SSTR1 somatostatin, and A1 adenosine) were coexpressed in COS-7 cells with either WTq or -6q. Transfected cells were then incubated with the appropriate agonist ligands, and the ability of the different receptors to couple to the two G proteins was determined by measuring increases in inositol phosphate production (due to WTq-mediated activation of PLC β ; Refs. 31 and 32). Coexpression of the different $G_{i/o}$ -coupled receptors with either vector DNA (pcDNA1) or WTq, followed by ligand stimulation, resulted only in a rather small increase in PLC β activity (Fig. 2A). As shown in Fig. 3 for the m2 muscarinic and D2 dopamine receptors, this small increase in inositol phosphate production could be almost completely blocked by pretreatment of cells with PTX (500 ng/ml). Consistent with previous findings (33, 34), this observation suggests that the m2 muscarinic and D2 dopamine receptors do not couple to WTq to a significant extent and that the small increase in PI hydrolysis seen after stimulation of these receptors is most likely due to activation of PLC β by G protein $\beta\gamma$ subunits released upon receptor-mediated activation of endogenous $G_{i/o}$ proteins (35, 36).

Interestingly, coexpression of the different $G_{i/o}$ -coupled receptors with -6q resulted in a significantly increased PI response (as compared with WTq) that was most pronounced in the case of the two biogenic amine receptors (m2 muscarinic and D2 dopamine; Fig. 2A). These responses could only be partly blocked by PTX pretreatment (shown for the m2 muscarinic and D2 dopamine receptors in Fig. 3), indicating that they were primarily due to receptor-mediated generation of activated -6q. Complete concentration-response curves for m2 muscarinic and D2 dopamine receptor-mediated activation of -6q are given in Fig. 4. The EC₅₀ values (means \pm S.E. of five independent experiments, each carried out in triplicate) for these responses amounted to $7.4 \pm 0.3 \mu$ M in the case of the

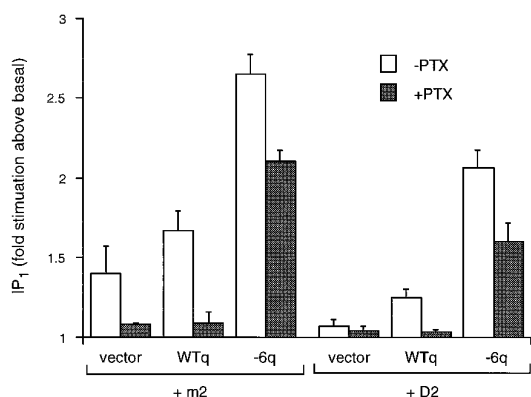


FIG. 3. Effect of pertussis toxin on inositol phosphate accumulation in cells cotransfected with WTq or -6q and $G_{i/o}$ -coupled receptors. COS-7 cells were cotransfected with expression plasmids coding for the wild type m2 or the D2 dopamine receptor and vector DNA (pcDNA1), WTq, or -6q. Transfected cells were incubated for 1 h (at 37 °C) in the absence or the presence of the appropriate agonist ligands. The resulting increases in intracellular IP_1 levels were determined as described under "Experimental Procedures," either in the absence or in the presence of PTX (500 ng/ml). Data are given as the means \pm S.E. of four independent experiments, each carried out in triplicate. The following ligands were used: m2 muscarinic receptor, carbachol (100 μ M); D2 dopamine receptor, (-)-quinpirole (10 μ M).

muscarinic agonist, carbachol (Fig. 4A), and to $1.4 \pm 0.2 \mu$ M in the case of the dopaminergic ligand, (-)-quinpirole (Fig. 4B), indicating that the interaction of the two biogenic amine receptors with -6q was highly efficient.

We next examined the ability of three G_s -coupled receptors (D1 dopamine, V2 vasopressin, and β_2 -adrenergic) to functionally interact with WTq or -6q. Consistent with their known coupling profiles (37), these receptors were unable to activate WTq to an appreciable extent (Fig. 2B). However, two of the investigated G_s -coupled receptors (D1 dopamine and β_2 -adrenergic) gained the ability to induce a significant increase in inositol production when coexpressed with -6q (as compared with WTq; Fig. 2B).

Two previous studies have shown that $G_{q/11}$ -coupled receptors such as the NK2 neurokinin (16) or the m1 muscarinic receptor (17) can activate -6q in a fashion identical to WTq. Our data therefore suggest that -6q, in contrast to qWT, can be activated by receptors that are members of all three major functional classes of GPCRs.

The maximum degree of PLC stimulation mediated by the bona fide $G_{q/11}$ -coupled m3 muscarinic receptor (7) amounted to 6–10-fold (data not shown), indicating that activation of -6q by different $G_{i/o}$ - and G_s -coupled receptors (most of which mediated a 2–6-fold stimulation of PLC activity; Fig. 2) was not optimal. This observation is consistent with the currently held notion that efficient receptor-G protein coupling involves multiple sites of contact between the receptor protein and the G protein heterotrimer (1–7).

It should also be noted that coexpressed -6q did not improve coupling of the V2 vasopressin receptor to PLC stimulation and that the absolute magnitude of responses mediated by -6q upon coexpression with the κ -opioid or the SSTR1 somatostatin receptor, respectively, was quite small (Fig. 2), indicating that -6q is not generally promiscuous. One possible reason for the observed differences in the ability of the studied $G_{i/o}$ - and G_s -coupled receptors to interact with -6q may be that the relative functional importance of individual receptor-G protein contact sites may vary among different GPCRs (e.g. peptide receptors versus receptors for biogenic amines).

Subcellular Distribution of WTq and -6q—To exclude the possibility that the promiscuous nature of -6q was simply due

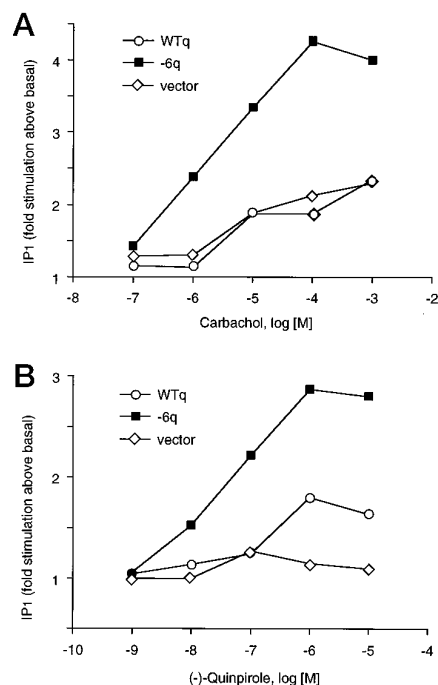


FIG. 4. Functional interaction of the m2 muscarinic and the D2 dopamine receptor with -6q. COS-7 cells cotransfected with vector DNA (pcDNA1), WTq, or -6q and m2 muscarinic (A) or D2 dopamine receptor (B) expression plasmids were incubated for 1 h (at 37 °C) with increasing concentrations of carbachol (A) or (-)-quinpirole (B). The resulting increases in intracellular IP_1 levels were determined as described under "Experimental Procedures." The results (mean values) from a representative experiment, carried out in triplicate, are shown; four additional experiments gave similar results.

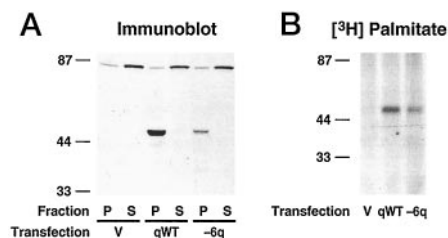


FIG. 5. Subcellular Localization and Palmitoylation of WTq and -6q. COS-7 cells were transfected with vector DNA alone (V), WTq, or -6q. A, cellular proteins were separated into particulate (P) and soluble (S) fractions, and $G\alpha$ subunits were detected by Western blotting using the HA epitope-specific 12CA5 antibody as described under "Experimental Procedures." B, following labeling of cells with [3 H]palmitic acid, proteins were immunoprecipitated with the 12CA5 antibody. The immunoprecipitates were resolved by SDS-PAGE and analyzed by fluorography as described under "Experimental Procedures." WTq and -6q proteins run at approximately 44 kDa. Protein molecular mass standards (in kDa) are indicated.

to exceptionally high expression levels (as compared with WTq), the subcellular distribution of the two G protein subunits was studied by cell fractionation and immunoblotting. Both $G\alpha$ subunits were detected with the monoclonal antibody, 12CA5, which recognizes the internal HA epitope tag present in both proteins (see "Experimental Procedures"). As shown in Fig. 5A, both G protein constructs were found exclusively in the particulate fraction; however, -6q was expressed at lower levels (approximately 10–20% of WTq, as determined by scanning densitometry; data not shown). The precise reason for this latter observation remains unclear; however, possible factors may include reduced protein stability or translation efficiency. In any case, these data exclude the possibility that the ability of -6q to be activated by multiple classes of GPCRs is due to

overexpression of this subunit (as compared with WTq).

Palmitoylation Pattern of WTq and -6q—Previous studies (16–18, 38–40) have shown that G proteins of the $G\alpha_{q/11}$ family, like most other $G\alpha$ subunits, are palmitoylated at cysteine residues located near the N terminus of the proteins (corresponding to Cys⁹ and Cys¹⁰ in Fig. 1). To compare the palmitoylation patterns of WTq and -6q, transfected COS-7 cells were metabolically labeled with [³H]palmitic acid followed by immunoprecipitation of WTq and -6q by the 12CA5 monoclonal antibody, SDS-PAGE, and fluorography. As shown in Fig. 5B, WTq and -6q were the only immunoprecipitated proteins, because no labeled proteins could be precipitated when cells were transfected with “empty” vector DNA. Consistent with published results, both WTq (16–18, 38, 39) and -6q (16) incorporated considerable amounts of [³H]palmitate (Fig. 5B). The reduction in signal strength seen with -6q (approximately 10–20% of WTq, as determined by scanning densitometry; data not shown) correlated well with the reduction in -6q levels revealed by immunoblotting (Fig. 5A). This observation suggests that -6q is palmitoylated to an extent similar to that of WTq, as has been observed earlier in transfected COS-7 cells (16). It is therefore unlikely that differences in palmitoylation patterns are responsible for the different functional properties of -6q and WTq.

Conclusions—We have shown that -6q, in contrast to WTq, can productively interact with several different $G_{i/o}$ - and G_s -coupled receptors, suggesting that the six-amino acid extension that is unique to WTq (as well as $G\alpha_{11}$) is critical for constraining the receptor coupling selectivity of this G protein subunit. -6q and WTq were found to be palmitoylated to a similar extent, suggesting that the functional promiscuity of -6q is not due to a lack of acylation. One possibility is that the N-terminal extension characteristic for $G\alpha_{q/11}$ subunits constrains the receptor coupling selectivity by preventing access of $G_{i/o}$ - and G_s -coupled receptors. Alternatively, it is conceivable that this six-amino acid sequence exerts an indirect conformational effect on the structure of $G\alpha_{q/11}$ that is crucial for maintaining the receptor selectivity of these subunits. Clearly, this issue needs to be addressed in future studies. In summary, our data suggest a novel mechanism by which receptor-G protein coupling selectivity can be achieved and should contribute to a better understanding of the molecular basis of receptor-G protein interactions.

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