Phorbol Ester Treatment Increases the Exocytic Rate of the Transferrin Receptor Recycling Pathway Independent of Serine-24 Phosphorylation

Timothy E. McGraw, Kenneth W. Dunn, and Frederick R. Maxfield
Department of Pathology, Columbia University College of Physicians and Surgeons, New York, New York 10032

Abstract. In Chinese hamster ovary (CHO) fibroblast cells the protein kinase C activating phorbol ester, phorbol myristate acetate (PMA), stimulates an increase in cell surface transferrin receptor (TR) expression by increasing the exocytic rate of the recycling pathway. The human TR expressed in CHO cells is similarly affected by PMA treatment. A mutant human TR in which the major protein kinase C phosphorylation site, serine 24, has been replaced with the non-phosphorylatable amino acid glycine has been constructed to investigate the role of receptor phosphorylation in the PMA induced up-regulation. The Gly-24-substituted receptor binds, internalizes, and recycles Tf. Furthermore, the altered receptor mediates cellular Fe accumulation from diferric-Tf, thereby fulfilling the receptor's major biological role. The Gly-24 TR behaves identically to the wild-type TR when cells are treated with PMA. Therefore, Ser-24 phosphorylation is not required for the PMA-induced redistribution of the human TR expressed in CHO cells. The increased TR expression on the cell surface after PMA treatment results from an increase in the rate of exocytosis of the recycling receptors. No change in the endocytic rate or the size of the recycling receptor pool was observed. These results indicate that the PMA effect on the TR surface expression may result from a more general perturbation of membrane trafficking rather than a specific modulation of the TR.

IRON uptake in mammalian cells is mediated by transferrin receptor (TR) endocytosis. Diferric transferrin (Tf) binds to the TR and is internalized by receptor-mediated endocytosis. Tf releases iron upon encountering an acidic intracellular vesicle pool. The apo-Tf-TR complex is recycled back to the cell surface to mediate further rounds of endocytosis (for review see Hanover and Dickson, 1985, and Huebers and Finch, 1987). The features of the TR responsible for internalization and recycling have not been identified.

The steady-state TR distribution between intracellular pools and the cell surface is altered by treatment with polypeptide hormones and chemical mitogens (Buys et al., 1984; Davis and Czech, 1986; Davis et al., 1986a; Fallon and Schwartz, 1986; Klausner et al., 1984; May et al., 1984; May et al., 1985; Tanner and Lienhard, 1987; Wiley and Kaplan, 1984). The effect is cell line and treatment dependent. For example, in the K562 human erythroleukemia cell line phorbol 12-myristate 13-acetate (PMA) causes a rapid decrease in cell surface TR expression (Klausner et al., 1984), whereas in mouse tumor macrophage-like cells, J774, PMA causes an increase in cell surface TR expression (Buys et al., 1984). Since PMA treatment also induces a reversible hyperphosphorylation of the TR, it has been proposed that TR phosphorylation is involved in regulating the TR distribution (Klausner et al., 1984; May et al., 1984). The cytoplasmic Ser-24 residue of the human TR has been identified as the site of PMA-activated protein kinase C phosphorylation (Davis et al., 1986b). By in vitro site-directed mutagenesis of the cDNA clone of the human TR the role of TR phosphorylation in regulation of TR distribution can be investigated.

Two groups have recently shown that Ser-24 phosphorylation is not required for endocytosis or recycling of the TR (Rothenberger et al., 1987, Zerial et al., 1987). In both studies the TR was transfected into cell lines expressing endogenous receptor. To follow the behavior of the transfected receptor, in the background of endogenous receptor, it was necessary to either express high levels of the transfected TR (approximately 10-fold over endogenous levels) or to use specific anti-human-TR antibodies as a marker for the TR. In neither case was it possible to study the TR behavior using Tf as a marker in clonal cell lines expressing a typical number of TR.

To circumvent the difficulties presented by the endogenous TR background we have developed a heterologous system in which to study the behavior of in vitro mutated human TR (McGraw et al., 1987). We have isolated TR-variant Chinese hamster ovary (CHO) cells (termed TRVb cells) that, due to a defect in the endogenous TR, do not bind detectable amounts of Tf. We have used these cell lines as recipients of a cDNA clone of the human TR and have shown that the hu-

1. Abbreviations used in this paper: PMA, 12-phorbol 13-myristate acetate; Tf, transferrin; TR, transferrin receptor.
Materials and Methods

**Cells and Cell Culture**

WTB cells were used as the wild-type CHO cells (Thompson and Baker, 1973). The isolation and characterization of the TR-variant CHO cell line, TRvb, and the TRvb cell line transfected with the human TR, TRvb-I, have been described previously (McGraw et al., 1987). Cells were maintained in Ham's nutrient F12 medium (Gibco, Grand Island, NY) supplemented with 5% FCS at 37°C in a humidified atmosphere of 5% CO2 in air. Transfectant cell lines were carried in medium containing 200 μg/ml G-418 (Gibco). Single cell colony lines were isolated by two rounds of cloning ring colony purification.

**Ligands**

Human Tf (Sigma Chemical Co., St. Louis, MO) was further purified by Sephacryl S-300 gel filtration. Differ Tf, fluorescein Tf, and [125I]Tf were prepared as previously described (Yamashiro et al., 1984). 35FeCl3 was purchased from New England Nuclear (Cambridge, MA). Tfn35Fe was prepared by the nitrilotriacetic acid method (Klausner et al., 1984). Fluorescein-Tf uptake and immunofluorescence were performed as described previously (McGraw et al., 1987). The human specific anti-TR monoclonal antibody B3/25 was purchased from Mannheim Biochemicals, Indianapolis, IN.

**Assays for PMA Effect on TR Expression**

Approximately 1 x 10^5 cells were plated per 35-mm well of 6 well plates 2 d before use. For surface-binding studies the cell monolayers were washed three times with 150 mM NaCl, 20 mM Hepes, pH 7.4, 1 mM CaCl2, 5 mM KCl, 1 mM MgCl2, 10 mM glucose), and incubated in medium for 30 min at 37°C in air. The cells were then treated with 100 μM PMA (Sigma Chemical Co.) for specified times of periods of time. The cells were then washed twice with ice cold medium, incubated with 2 μg/ml [125II]Tf, 2 mg/mi ovalbumin at 4°C for 2 h. The cells were washed five times at 4°C with medium 1, solubilized with 0.1% Triton X-100 in 0.1 N NaOH, and counted in a gamma counter. Nonspecific binding was determined by incubation in the presence of a 500-fold excess of unlabelled Tf. Nonspecific binding was generally less than 5% of total binding. Each experiment consisted of three experimental and three competition wells.

The Tf washout assay was performed by washing cell monolayers twice with medium 1 and incubating with 2 μg/ml [125II]Tf, 2 mg/mi ovalbumin for 60 min at 37°C. The cells were rapidly washed three times with medium 1 containing 50 μM desferoxamine (Ciba-Geigy Corp., Greensboro, NC) and further incubated in medium 1 containing 50 μM desferoxamine with or without 100 nM PMA. At the specified times the medium was aspirated and the cells were incubated on ice with 280 mM sucrose, 50 mM MES (pH 5.0) for 10 min. This acid wash insures that all surface bound [125II]Tf is removed. The cells were then washed with ice cold medium 1 and cell-associated radioactivity determined after solubilization of the monolayers. Desferoxamine was included in the incubations to ensure that apo-Tf released from the cell was not re-iron loaded.

**Construction of Mutant TR**

The ∼4.9 kb Bam HI insert fragment of the cDNA clone of the human TR pCDTDRI (McClelland et al., 1984) was transferred to the Bam HI site of a pUC8 plasmid derivative in which the Hind III site had been eliminated. In this construct, pTM1008, the insert is oriented with the 5' end of the message adjacent to the Eco RI site of pUC8. pTM1008 contains two Hind III sites, one at 916 bp of the coding region of the insert and the other at ~3.8 kb of the non-coding region of the cDNA clone (McClelland et al., 1984). pTM1008 was partially digested with Hind III and the linearized fragment (~7.7 kb) was gel purified, the Hind III ends were filled-in by treatment with Klenow, blunt-end ligated and transformed into *Escherichia coli* strain JM109. Single colonies were purified and the DNA analysed by restriction digestion. A clone was selected in which the Hind III site in the non-coding region had been inactivated. This construct, pTM1100, retained the Hind III site in the coding region. The ∼1 kb Eco RI (site in pUC8 sequences)-Hind III fragment of pTM1100 was transferred to Eco RI-Hind III digested M13mp8. This fragment contains the coding sequences for the NH2 terminal 273 amino acids of the TR, including the 61 amino-acid NH2 terminal cytoplasmic tail, the putative membrane-spanning region and 183 amino acids of the extracellular portion of the receptor. A synthetic oligonucleotide, 3'-GGGGCAAGCGGACC-5', in which the codon for Ser-24 was replaced with the codon for Gly was used as primer for in vitro site-directed mutagenesis after the procedures of Zoller and Smith (1982) with the modifications of Kunkel (1985). The nucleotide change was confirmed by Sanger sequencing across the mutated site. The mutated Eco RI-Hind III fragment was isolated from the replicative form of M13 and was reinserted into pTM1100 replacing the wild-type Eco RI-Hind III fragment. Transfection of TRvb cells was performed as previously described (McGraw et al., 1997). For transfection the Bam HI cDNA insert was mixed with Bam HI vector fragment of pCDTDRI and uncut pSV3Neo. The cDNA plasmid, pCDTRI, only gives transfectants when the insert is released by Bam HI digestion (McGraw et al., 1987). We found that inclusion of the Bam HI vector fragment of pCDTRI increases the TR positive transfection frequency. In this transfection scheme the promoter used for TR expression is unknown.

**Results**

**PMA Effects an Increase in Cell Surface TR Expression in CHO Cells**

Treatment of CHO cells with PMA causes a rapid increase in cell surface TR expression, as measured by an increase in 125I-Tf binding at 4°C (Fig. 1). After a 5-min incubation with 100 nM PMA, 125I-Tf surface binding increases by 30–50%. The effect is complete within 5 min, as longer incubations with PMA have no further effect on 125I-Tf surface binding. Since CHO cells respond to PMA treatment by increasing the exocytic rate of the TR recycling pathway. Phosphorylation of Ser-24 is not required for the PMA effect since the Gly-24 substituted receptor is similarly affected by PMA treatment.

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protein kinase C, did not alter the cell surface TR expression binding to TRVb-1 cells treated for 10 min with 100 nM PMA and 4-alpha-phorbol, a phorbol ester which does not activate of the TR Recycling Pathway cant change in the dissociation constant. In control cells the revealed a 35% increase in surface binding with no signifi-
Figure 1. Time course of the PMA effect on cell surface TR expression in CHO cells. CHO cells were treated with 100 nM PMA in med 1 at 37°C for 5-30 min. The cells were cooled to 4°C, and [125I]Tf-binding was measured. The results shown are the mean ± SEM of [125I]Tf-binding relative to untreated cells in four separate experiments.
nM 4-alpha-phorbol, a phorbol ester which does not activate protein kinase C, did not alter the cell surface TR expression (not shown).

PMA treatment of TRVb-1 cells does not alter the affinity of the human TR for Tf. Scatchard analysis of [125I]Tf 4°C binding to TRVb-1 cells treated for 10 min with 100 nM PMA revealed a 35% increase in surface binding with no signifi-
cant change in the dissociation constant. In control cells the Kd was measured as 1.50 ± 0.1 nM; and after pretreatment with 100 nM PMA for 10 min the Kd was measured as 1.48 ± 0.02 nM Kd (mean ±SD of three experiments).

PMA Stimulates an Increase in the Exocytic Rate of the TR Recycling Pathway

Since the PMA effect on surface expression of TR is rapid it was unlikely that the effect was a result of the synthesis of new receptors. Rather, it was more likely that PMA was in-
ducing a redistribution of existing TR. Surface expression could be increased by recruitment to the cell surface of intra-
cellular TR either from the recycling pool of receptors (that is, altering the kinetics of recycling) or from a distinct pool of noncycling receptors (that is, increasing the size of the recycling pool). To distinguish between these possibilities the size of the recycling pool of TR was measured. TRVb-1 cells were incubated for 60 min at 37°C with 2 μg/ml [125I]Tf to equilibrate the recycling pools of TR with [125I]Tf. The cells were then incubated for an additional 15 min in 2 μg/ml [125I]Tf with or without 100 nM PMA. The cells were washed and total cell-associated [125I]Tf was determined. There was no difference in total cell-associated [125I]Tf between cells treated with PMA and control cells. This result suggests that PMA is inducing a redistribution of TR in the recycling pool, since the total number of recycling TR was not increased by PMA treatment.

Increased cell surface TR expression could result from an increase in the exocytic rate (movement of intracellular receptors back to the cell surface) or a decrease in the rate of internalization. Since apo-Tf returns to the cell surface with the TR it is possible to characterize the rate of return of the TR to the cell surface by following the release of apo-Tf from the cell. To examine the effect of PMA treatment on the exocy-
totic rate, cells were preincubated with [125I]Tf to equili-
brate the intracellular recycling pool of receptors with Tf. The cells were then washed free of unbound Tf and further incubated with or without 100 nM PMA. At various times the cells were washed to remove any released apo-Tf, and cell-associated radioactivity was determined. PMA treatment results in an increase in the rate of externalization of apo-Tf (Fig. 3). The calculated rates are presented in Table I. The rate of return of the human TR expressed in TRVb-1 cells is increased by a factor of 1.36 ± 0.05 (mean ± SD of four separate determinations). PMA affects the exocytosis of the hamster TR similarly, increasing the exocytic rate by a factor of about 1.50 ± 0.2 (mean ±SD of three separate de-
terminations; Fig. 3, Table I). We have reported previously that the basal recycling rate of the hamster TR is faster than that of the human TR expressed in TRVb cells (Table I, McGraw et al., 1987). The increase in the rate of exocytosis correlates well with the increase in cell surface TR expression observed after PMA treatment (Figs. 1 and 2), suggesting that the increased surface expression is due to the effect on the recycling rate.

Figure 2. PMA effect on cell surface expression of the human TR in TRVb-1 cells. TRVb-1 is a CHO cell line, deficient in the expression of functional hamster TR, which expresses a cDNA clone of the human TR. A is a time course of the PMA effect on surface expression of the human TR. The cells were treated with 100 nM PMA in medium 1 at 37°C. The cells were cooled to 4°C and [125I]Tf-binding was measured. The results shown are the mean ± SEM of three separate determinations of [125I] Tf-binding relative to untreated cells. B is a dose-respon-
ses of the PMA effect on cell surface TR expression. The cells were treated with PMA for 10 min. Surface [125I]Tf-binding was determined as in A. The results presented are the means of three determinations ± SEM from a representive experiment.

Figure 3. PMA effect on TR exocytosis in TRVb-1 and CHO cells. The rates of [125I]Tf release from TRVb-1 (A) and CHO cells (B) were calcu-
lated as described in Materials and Methods. The percent of total cell-associated [125I]Tf remaining is plotted vs. time. The results presented are of representative experiments and are the means of triplicate determinations. A are TRVb-1 cells: (c) no treatment; (■) 100 nM PMA treatment. B are CHO cells: (c) no treatment; (■) 100 nM treatment.
The effect of PMA on the internalization rate of Tf was examined using the steady-state procedure of Wiley and Cunningham (1982). In two separate experiments the rate of [125I]Tf internalization in TRVb-1 cells was not significantly affected by PMA treatment. Together with the measured effect on exocytosis and the lack of a change in the size of the recycling receptor pool, these data show that the increased TR surface expression is a result of an increased rate of exocytosis.

**TR Ser-24 Phosphorylation is not Required for Receptor Functioning**

PMA-activated protein kinase C has been shown to phosphorylate the human TR on the cytoplasmic residue Ser-24 (Davis et al., 1986b). To investigate whether Ser-24 phosphorylation is required for the PMA induced redistribution of the human TR expressed in CHO cells, we constructed an in vitro mutagenized receptor in which Ser-24 has been replaced with the nonphosphorylatable amino acid, Gly. The mutated cDNA clone was transfected into TRVb cells and two independently derived cell lines were isolated: TRVb-Gly24A and TRVb-Gly24B.

The Gly substitution for Ser had no major effect on the behavior of the transfected receptor. Scatchard analysis of [125I]Tf binding at 4°C to these cell lines demonstrated that the Gly substitution did not affect the affinity of the TR for Tf (Table II). The two transfected cell lines express different numbers of the human TR, although neither varies greatly from the 60,000 hamster TR expressed in CHO cells.

Both the patterns of internalization of fluorescein-labeled Tf and the immunofluorescent localization of the receptor with the anti-human TR receptor monoclonal antibody B3/25 demonstrate that the Gly-24 TR is processed by the cell in a fashion indistinguishable from that of the wild-type receptor at the level of resolution of light microscopy (not shown).

The exocytic rate of the recycling pathway is shown in Table I for the two cell lines containing the altered receptor. [125I]Tf internalized by the two cell lines expressing the Gly-24 receptor is recycled and released from the cells with kinetics somewhat faster than that of the wild type receptor expressed in TRVb-1 cells (Table I). We do not know if the variation in basal exocytic rates in the cell lines shown in Table I reflects clonal variation among cell lines or if there is some receptor sequence effect.

The Gly-substituted receptor functions in delivery of iron to the cells. The mutant receptors accumulate 59Fe from [59Fe2]Tf at a rate approximately proportional to the level of receptor expression (Fig. 4). Since the Gly substitution does not affect the behavior of the TR in any of the above respects, it can be concluded that Ser-24 phosphorylation is not required for the normal functioning of the TR.

Although Ser-24 phosphorylation is not required for the normal functioning of the receptor, it could be required for the PMA-induced redistribution. To investigate this possibility, the effect of 100 nM PMA on the exocytic rate of the Gly-

### Table I. The Effect of PMA Treatment on the Exocytic Rates of the Recycling Pathway of TR

| Cell line | TR expressed | Exocytic rate of recycling |
|-----------|--------------|---------------------------|
| CHO       | Hamster TR   | 0.060 ± 0.006             |
| TRVb-1    | Human TR (Ser-24) | 0.032 ± 0.003 |
| TRVb-GlyA | Human TR (Gly-24) | 0.042 ± 0.002 |
| TRVb-GlyB | Human TR (Gly-24) | 0.040 ± 0.003 |

The exocytic rates were determined by calculating the slope of a plot of the log of the loss of cell-associated [125I]Tf vs. time. The values presented are the mean ± SEM of at least three separate experiments.

### Table II. Dissociation Constants and Surface Expression of Cell Lines Transfected with Wild Type and Mutant Human TR

| Cell line | Kd (nM) | Receptors/cell |
|-----------|---------|----------------|
| TRVb-1    | 1.5 ± 0.1 | 140,000 ± 12,000 |
| TRVb-GlyA | 2.4 ± 0.7 | 35,000 ± 10,000 |
| TRVb-GlyB | 1.4 ± 0.3 | 90,000 ± 10,000 |

The dissociation constants and surface receptor expression were calculated by Scatchard analysis of 4°C [125I]Tf binding. The results represent the mean ± SD of at least three different experiments.
orthophosphate for 4 h. For PMA treatment 100 nM PMA was added for the final 30 min of the labeling incubation. The cells were lysed and an equal number of TCA precipitable counts from each lysate were used for the immunoprecipitation. The TR was precipitated with the human TR specific monoclonal antibody B3/25 after a previously described procedure (Lipsich et al., 1983). Precipitated protein from each lysate was loaded per lane of the gel. PMA treatment stimulates phosphorylation of the wild-type human TR expressed in TRVb-1 cells. As expected the Gly-24 substitution greatly reduced PMA-stimulated TR phosphorylation. In TRVb-Gly24A cells no phosphorylation of the TR is observed (Fig. 6). There is, however, a small amount of PMA-stimulated phosphorylation of the TR in TRVb-Gly24B cells (not visible in Fig. 6). This result suggests that there may be other minor site(s) of TR phosphorylation when expressed in TRVb cells. This minor phosphorylation is not a universal characteristic of expression of the human Gly-24 TR in TRVb cells since in cell line TRVb-Gly24A no phosphorylation is detected, even when the gels were over exposed to account for differences in TR number. This result confirms our conclusion that the phosphorylation of Ser-24 is not required for the PMA-induced TR redistribution.

Discussion

The surface expression of TR is regulated both pre- and posttranslationally. Receptor expression is decreased when cells are treated with Tf-alternative iron sources such as iron salts or hemin, and it is increased by chelating intracellular iron (Bridges and Cudkowicz, 1984; Ward et al., 1984; Rao et al., 1985). Iron-dependent regulation of TR expression is reflected by changes in mRNA concentration, suggesting that the alteration in receptor expression is regulated at the level of receptor synthesis (Rao et al., 1986). TR surface expression is also regulated posttranslationally. In this report we show that in CHO cells, the tumor promoting phorbol ester PMA causes an increase in the cell surface expression of the TR. PMA affects the steady-state distribution of TR in CHO cells by inducing an increase in the rate of exocytosis of the recycling receptor. PMA has been shown to have a similar effect on the TR in mouse tumor macrophage-like cells, J774 (Buys et al., 1984). The PMA effect is, however, cell line dependent. In K562 and HL60 cells PMA causes a decrease in surface expression by stimulating internalization of the TR (Klausner et al., 1984; May et al., 1984; May et al., 1985). Insulin and epidermal growth factor cause an increase in cell surface TR expression in fat cells and human fibroblasts (Davis et al., 1986a; Wiley and Kaplan, 1984). Recently, it has been shown that insulin increases the rate of recycling of the TR in adipocyte cells (Tanner and Lienhard, 1987). Thus, surface expression of the TR is under complex regulation both pre- and posttranslationally.

An attractive proposal for posttranslational regulation was that a modification of the TR resulted in the observed steady-state redistribution of intracellular and surface receptors. Since the protein kinase C activating phorbol ester PMA is capable of inducing a redistribution and the human TR is phosphorylated by protein kinase C, it was possible that TR phosphorylation was responsible for the observed effects. By characterizing the behavior of the human TR in which the major protein kinase C phosphorylation site, Ser-24,
replaced with a non-phosphorylatable amino acid, Gly, we have shown that phosphorylation of Ser-24 is not required for the PMA-induced redistribution. Furthermore, the Gly-24 TR behaved similarly to the wild type Ser-24 receptor in all aspects of endocytosis examined, suggesting that Ser-24 phosphorylation is not required for the functioning of the receptor. TRVb cells do not proliferate when the only Fe source is dextran-Tf, presumably because of the lack of endogenous TR. The TRVb-Gly24 transfected cell lines do proliferate under these growth conditions, which indicates the complete biological functioning of the Gly-24 TR. Although our results demonstrate that Ser-24 phosphorylation is not required for the PMA effect on human TR expressed in CHO cells, it is still possible that phosphorylation of Ser-24 is involved in the growth factor-induced increase in TR surface expression or in the PMA-induced TR internalization in K562 and HL60 cells.

Our results suggest that phosphorylation of protein(s) other than the TR is responsible for regulating the trafficking of this membrane protein. The increased exocytic rate could result from a general increase in the rate of fusion of the recycling vesicles with the plasma membrane or from an increase in the rate of movement of the intracellular vesicles to the site of fusion with the cell membrane. Identifying the intracellular location of the action of PMA treatment requires a detailed morphological study of the exocytic portion of the TR recycling pathway.

Our results raise the question as to whether the PMA effect is specific for the TR (or a subclass of membrane proteins of which the TR is a member) or whether the TR redistribution reflects a general perturbation of cellular membrane traffic. Further characterization of a number of recycling membrane proteins in one cell system will be required to answer this question.

A report on the requirement of TR phosphorylation for endocytosis of the receptor has recently appeared (Rothenberger et al., 1987). Our result that Ser-24 phosphorylation is not required for basal functioning of the TR is in agreement with their results. In their study they have expressed the human TR in mouse LTK- cells, which do not respond to PMA treatment. They were, therefore, unable to investigate the role of TR phosphorylation in response to PMA. In a more recent study it has been shown that PMA up-regulates cell surface TR expression in mouse 3T3 cells and that this up-regulation is independent of TR phosphorylation (Zerial et al., 1987). These results are also consistent with our results. In our study we have taken advantage of a heterologous system lacking endogenous TR. This has allowed us to characterize the up-regulation of TR in detail using the native ligand.

This heterologous system is ideal for future morphological and biochemical studies of the exocytic portion of the TR recycling pathway directed at identifying the site of action of PMA on steady-state receptor distribution.

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**Note added in proof:** Similar results to those reported here have recently been published by R. J. Davis and H. Meisner 1987, J. Biol. Chem. 262: 16041-16047.

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