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

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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)[1] 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 apoTf-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., 1986*a*; 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., 1986*b*). 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.

man TR expressed in TRVb cells behaves similarly to the hamster TR in wild-type CHO cells. The advantage of using this system for structure/function studies of the TR is that the behavior of the receptor can be characterized without interference from endogenous TR activity. This allows the use of Tf as a marker for the receptor in cell lines expressing a typical number of transfected TR. Furthermore, the biological function of the transfected receptors (that is, uptake of Fe via Tf) can be directly assessed without interference from endogenous receptor.

In this paper we report the characterization of the behavior of the human TR in which Ser-24 has been replaced with the nonphosphorylatable amino acid, Gly. The mutant receptor binds Tf, internalizes, and recycles similarly to the wild-type human receptor expressed in TRVb cells. We also show that PMA induces a rapid increase in cell surface expression of the hamster TR in CHO cells, as well as the human TR expressed in TRVb cells, 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.

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-1, 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% CO_2 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. Differic Tf, fluorescein Tf, and [^{125}I]Tf were prepared as previously described (Yamashiro et al., 1984). $^{59}FeCl_3$ was purchased from New England Nuclear (Cambridge, MA). $Tf^{59}Fe_2$ 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 1×10^5 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 med 1 (150 mM NaCl, 20 mM Hepes, pH 7.4, 1 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose), and incubated in med 1 for 30 min at 37°C in air. The cells were treated with 100 nM PMA (Sigma Chemical Co.) for specified periods of time. The cells were then washed twice with ice cold med 1, incubated with 2 μg/ml [^{125}I]Tf, 2 mg/ml ovalbumin at 4°C for 2 h. The cells were washed five times at 4°C with med 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 unlabeled 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 med 1 and incubating with 2 μg/ml [^{125}I]Tf, 2 mg/ml ovalbumin for 60 min at 37°C. The cells were rapidly washed three times with med 1 containing 50 μM desferroxamine (Ciba-Geigy Corp., Greensboro, NC) and further incubated in med 1 containing 50 μM desferroxamine 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 [^{125}I]Tf is removed. The cells were then washed with ice cold med 1 and cell-associated radioactivity determined after solubilization of the monolayers. Desferroxamine 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 pCDTR1 (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 911 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, pTM1010, retained the Hind III site in the coding region. The ~1 kb Eco RI (site in pUC8 sequences)-Hind III fragment of pTM1010 was transferred to Eco RI-Hind III digested M13mp18. This fragment contains the coding sequences for the NH_2 terminal 273 amino acids of the TR, including the 61 amino-acid NH_2 terminal cytoplasmic tail, the putative membrane-spanning region and 183 amino acids of the extracellular portion of the receptor. A synthetic oligonucleotide, 3′-GGGCCAAGCCGGACCG-5′, in which the codon for Ser-24 has been 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 pTM1010 replacing the wild-type Eco RI-Hind III fragment. Transfection of TRVb cells was performed as previously described (McGraw, et al., 1987). For transfection the Bam HI cDNA insert was mixed with Bam HI vector fragment of pCDTR1 and uncut pSV3-Neo. The cDNA plasmid, pCDTR1, 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 pCDTR1 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 [^{125}I]Tf binding at 4°C (Fig. 1). After a 5-min incubation with 100 nM PMA, [^{125}I]Tf surface binding increases by 30–50%. The effect is complete within 5 min, as longer incubations with PMA have no further effect on [^{125}I]Tf surface binding. Since CHO cells respond to PMA treatment by modulating the cell surface expression of the TR, these cells can be used as a recipient cell line for studying the role of phosphorylation in regulating cell surface TR expression.

We have isolated TR-variants of CHO cells (TRVb) which do not express detectable TR (McGraw et al., 1987). We have transfected a cDNA clone of the human TR into TRVb cells (the transfected cell line termed TRVb-1) and have shown that the transfected human TR behaves similarly to the hamster TR (McGraw et al., 1987). The cell surface expression of the human TR of TRVb-1 cells is rapidly increased after PMA treatment, in a fashion similar to the up-regulation of the hamster TR in CHO cells (Fig. 2 *a*). This PMA effect is dose-dependent, with a half-maximal dose of ~20 nM (Fig. 2 *b*). The PMA effect appears to be mediated by protein kinase C activation since treatment of TRVb-1 cells with 100

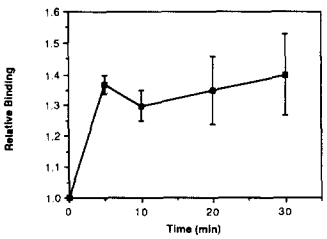

Figure 1. Time course of the PMA effect on cell surface TR expression in CHO cells. CHO cells were treated with 100 nM PMA in med 1 at 37°C for 5–30 min. The cells were cooled to 4°C, and [^{125}I]Tf-binding was measured. The results shown are the mean ± SEM of [^{125}I]Tf-binding relative to untreated 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 [^{125}I]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 significant change in the dissociation constant. In control cells the K_d was measured as 1.50 ± 0.1 nM; and after pretreatment with 100 nM PMA for 10 min the K_d was measured as 1.48 ± 0.02 nM K_d (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 inducing a redistribution of existing TR. Surface expression could be increased by recruitment to the cell surface of intracellular 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 [^{125}I]Tf to equilibrate the recycling pools of TR with [^{125}I]Tf. The cells were then incubated for an additional 15 min in 2 μg/ml [^{125}I]Tf with or without 100 nM PMA. The cells were washed and total cell-associated [^{125}I]Tf was determined. There was no difference in total cell-associated [^{125}I]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 exocytic rate, cells were preincubated with [^{125}I]Tf to equilibrate 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 determinations; 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.

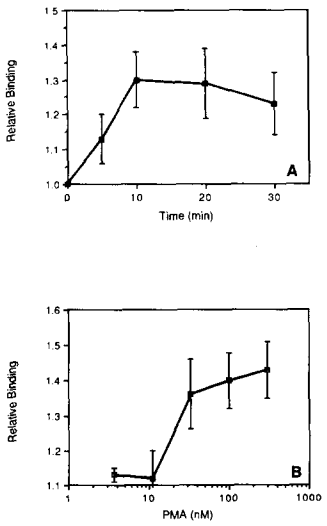

Figure 2. PMA effect on cell surface expression of the human TR in TRVb-1 cells. TRVb-1 is a CHO cell line, deficient in the expression of functional hamster TR, which expresses a cDNA clone of the human TR. *A* is a time course of the PMA effect on surface expression of the human TR. The cells were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

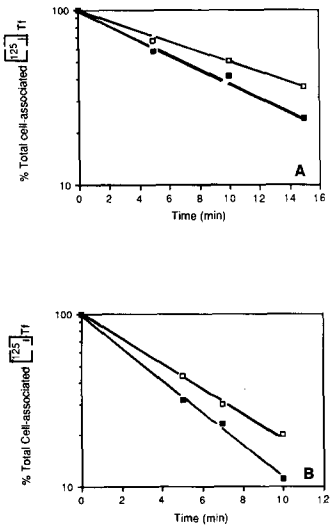

Figure 3. PMA effect on TR exocytosis in TRVb-1 and CHO cells. The rates of [^{125}I]apoTf release from TRVb-1 (*A*) and CHO cells (*B*) were calculated as described in Materials and Methods. The percent of total cell-associated [^{125}I]Tf remaining is plotted vs. time. The results presented are of representative experiments and are the means of triplicate determinations. *A* are TRVb-1 cells: (□) no treatment; (■) 100 nM PMA treatment. *B* are CHO cells: (□) no treatment; (■) 100 nM treatment.

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 min^{-1}	
		No PMA	*100 nM PMA*
CHO	Hamster TR	0.060 ± .006	0.090 ± .001
TRVb-1	Human TR (Ser-24)	0.032 ± .003	0.044 ± .002
TRVb-GlyA	Human TR (Gly-24)	0.042 ± .002	0.053 ± .004
TRVb-GlyB	Human TR (Gly-24)	0.040 ± .003	0.051 ± .001

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

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 [^{125}I]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., 1986*b*). 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 [^{125}I]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).

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

Cell line	K_d	Receptors/cell
	nM	
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 [^{125}I]Tf binding. The results represent the mean ±SD of at least three different experiments.

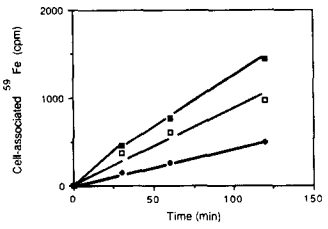

Figure 4. ^{59}Fe accumulation in TRVb-1, TRVb-GlyA, and TRVb-GlyB cells. TRVb-1 (■), TRVb-GlyA (♦), TRVb-GlyB (□), cells were incubated with 2 μg/ml Tf^{59}Fe$_2$ at 37°C. Cell-associated ^{59}Fe was determined by solubilizing the monolayers after washing. The data is presented as ^{59}Fe accumulation (cpm) per 1 ± 10^6 cells. Nonspecific ^{59}Fe accumulation was determined by incubation in the presence of a 500-fold excess of unlabeled Tf.

The exocytic rate of the recycling pathway is shown in Table I for the two cell lines containing the altered receptor. [^{125}I]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 ^{59}Fe from [^{59}Fe$_2$]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-

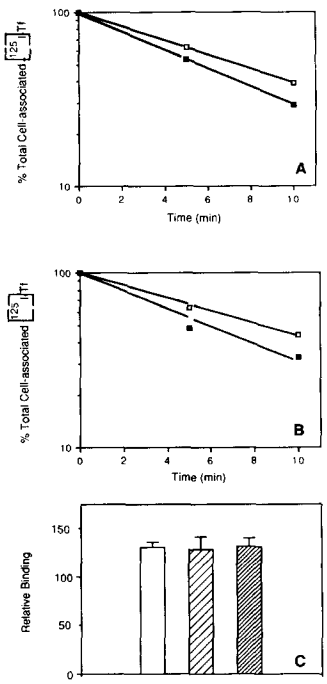

Figure 5. PMA effect on the exocytosis of Gly-24 substituted human TR. The rates of [^{125}I]apoTf release from TRVb-Gly24A and TRVb-Gly24A cells were calculated as described in Materials and Methods. The percent of total cell-associated [^{125}I]Tf remaining is plotted vs. time. The results presented are of representative experiments and are the means of triplicate determinations. *A* are TRVb-GlyA cells: (□) no treatment; (■) 100 nM PMA treatment. *B* are TRVb-GlyB cells: (□) no treatment; (■) 100 nM treatment. (*C*) PMA effect on TR surface expression measured by [^{125}I]Tf-binding at 4°C. The cells, □, TRVb-1; ▨, TRVb-Gly24A; ▨, TRVb-Gly24B; were incubated with 100 nM PMA for 10 min then cooled to 4°C and incubated with 2 μg/ml [^{125}I]Tf for 2 h. Binding after PMA treatment is presented relative to control binding. The results are the means of three experiments ± SD.

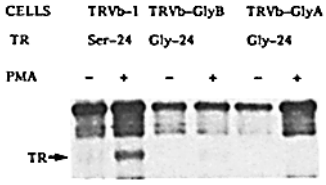

Figure 6. Immunoprecipitation of PMA-stimulated phosphorylation of the wild-type human TR from TRVb-1, and the Gly-24-substituted receptor from TRVb–Gly24A and TRVb–Gly24B cells. Cells were incubated with 1.0 mCi/ml [^{32}P]orthophosphate for 4 h. For PMA treatment 100 nM PMA was added for the final 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). 50 μM Na_3VO_4 was present in the lysis and immunoprecipitation buffers to inhibit phosphatases. After boiling the immunoprecipitates in sample buffer, one-half (by volume) of the total immunoprecipitated protein from each lysate was loaded per lane of a 7.5% SDS-polyacrylamide gel under reducing conditions. To insure that all the TR was detected the beads were not extensively washed. This results in an increase in nonspecific binding to the beads. The nonspecific pattern was identical whether or not B3/25 was included in the immunoprecipitation.

mutated TR was examined. In both cell lines PMA treatment results in an increase in the rate of Tf release (Fig. 5; Table I). In TRVb–Gly24A there was a 1.32 ± .05 fold (mean ± SD) and in TRVb-Gly24B a 1.26 ± .02 fold (mean ± SD of four separate determinations) increase in the rate of Tf release. Furthermore, the surface TR expression, measured by [^{125}I]Tf binding at 4°C, is increased 30–40% by 100 nM PMA treatment (Fig. 5 *C*). These results demonstrate that Ser-24 phosphorylation is not required for the PMA-induced increase in the exocytic rate of the human TR expressed in TRVb cells.

The human TR is a disulphide linked homodimer of 96,000-D chains (Trowbridge et al., 1984). Since the receptors is a dimer it is possible that heterodimers between the transfected receptor and the endogenous receptor could form when the cDNA clone is expressed in a heterologous cell system. The TR-variant CHO cell line used in this study, TRVb cells, is devoid of functional endogenous TR. However, we have not characterized the mutation responsible for this phenotype. It is possible that TRVb cells express hamster TR which are mutated in the Tf-binding site. If this were the case, it would be possible to form heterodimers between the human TR monomer, introduced by transfection, and the mutant hamster receptor. In such a heterodimer the hamster cytoplasmic portion could complement the site-directed mutation in human receptor. Although we cannot directly rule out this possibility, we think that it is unlikely for several reasons. If heterodimers were formed, we would expect that the proportion of heterodimers in the transfected cell lines would be substantially different from one another due to the different amounts of transfected TR expressed in each cell line (35,000 and 90,000 TR per cell). However, in all measured respects the receptors behave identically in both Gly-24 transfectants. Therefore we do not believe that heterodimers are masking an effect of the Gly-24 substitution on the functioning of the TR.

To confirm that the Gly-24 substitution abolishes receptor phosphorylation, the TR was immunoprecipitated from TRVb-1, TRVb-Gly24A, and TRVb-Gly24B cell lines preincubated with [^{32}P]-orthophosphate and treated with 100 nM PMA for 15 min (Fig. 6). To account for any differences among the cell lines in the activity of protein kinase C or the incorporation of ^{32}P into ATP pools, an equal number of TCA precipitable counts from each lysate were used in the immunoprecipitation. Equal aliquots (by volume) of sample buffer containing total immunoprecipitated protein from each lysate were 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., 1986*a*; 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, was

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 differic-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 if 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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References

Bridges, K. R., and A. Cudkowicz. 1984. Effect of iron chelators on the transferrin receptor in K562 cells. *J. Biol. Chem.* 259:12970-12977.

Buys, S. S., E. A. Keogh, and J. Kaplan. 1984. Fusion of intracellular membrane pools with cell surfaces of macrophages stimulated by phorbol esters and calcium ionophores. *Cell.* 38:569-576.

Davis, R. J. and M. P. Czech. 1986. Regulation of transferrin receptor expression at the cell surface by insulin-like growth factors, epidermal growth factor, and platelet-derived growth factor. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:653-658.

Davis, R. J., S. Corvera, and M. P. Czech. 1986*a*. Insulin stimulates cellular iron uptake and causes a redistribution of intracellular transferrin receptors to the plasma membrane. J. Biol. Chem. 261:8708-8711.

Davis, R. J., G. L. Johnson, D. J. Kelleher, J. K. Anderson, J. E. Mole, and M. P. Czech. 1986*b*. Identification of serine 24 as the unique site on the transferrin receptor phosphorylated by protein kinase C. *J. Biol. Chem.* 261: 9034-9041.

Fallon, R. J., and A. L. Schwartz. 1986. Regulation by phorbol esters of asialoglycoprotein and transferrin receptor distribution and ligand affinity in a hepatoma cell line. *J. Biol. Chem.* 261:15081-15089.

Hanover, C. J., and R. B. Dickson. 1985. Transferrin: receptor-mediated endocytosis and iron delivery. *In* Endocytosis. I. Pastan and M. C. Willingham, editors. Plenum Publishing Corp., New York. 131-162.

Huebers, H. A., and C. A. Finch. 1987. The physiology of transferrin and transferrin receptors. *Physiological Rev.* 67:520-582.

Klausner, R. D., J. Harford, and J. van Renswoude. 1984. Rapid internalization of the tranferrin receptor in K562 cells is triggered by ligand binding or treatment with a phorbol ester. *Proc. Natl. Acad. Sci. USA.* 81:3005-3009.

Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotype selection. *Proc. Natl. Acad. Sci. USA.* 82:488-492.

Lipsich, L. A., A. J. Lewis, and J. S. Brugge. 1983. Isolation of monoclonal antibodies that recognize the transforming proteins of avian sarcoma virus. *J. Virol.* 48:352-360.

May, W. S., S. Jacobs, and P. Cuatrecasas. 1984. Association of phorbol ester induced hyperphosphorylation and reversible regulation of transferrin membrane receptors in HL60 cells. *Proc. Natl. Acad. Sci. USA.* 81:2016-2020.

May, W. S., N. Sahyoun, S. Jacobs, M. Wolf, and P. Cuatrecasas. 1985. Mechanism of phorbol-induced regulation of surface transferrin receptor involves the action of activated protein kinase C and intact cytoskeleton. *J. Biol. Chem.* 260:9419-9426.

McClelland, A. L., L. C. Kuhn, and F. H. Ruddle. 1984. The human transferrin receptor gene: genomic organization, and the complete primary structure of the receptor deduced from a cDNA sequence. *Cell.* 39:267-274.

McGraw, T. E., L. Greenfield, and F. R. Maxfield. 1987. Functional expression of the human transferrin receptor cDNA in Chinese hamster ovary cells deficient in endogenous transferrin receptor. *J. Cell Biol.* 105:207-214.

Rao, K., D. Shapiro, E. Mattia, K. Bridges, and R. Klausner. 1985. Effects of alterations in cellular iron on biosynthesis of the transferrin receptor in K562 cells. *Mol. Cell. Biol.* 5:595-600.

Rao, K., J. B. Harford, T. Rouault, A. McClelland, F. H. Ruddle, and R. D. Klausner. 1986. Transcriptional regulation by iron of the gene for the transferrin receptor. *Mol. Cell. Biol.* 6:236-240.

Rothenberger, S., B. J. Iacopetta, and L. C. Kuhn. 1987. Endocytosis of the transferrin receptor requires the cytoplasmic domain but not its phosphorylation site. *Cell.* 49:423-431.

Tanner, L. I., and G. E. Lienhard. 1987. Insulin elicits a redistribution of transferrin reeptors in 3T3-LI adipocytes through an increase in the rate constant for receptor externalization. *J. Biol. Chem.* 262:8975-8980.

Thompson, L. H., and R. M. Baker. 1973. Isolation of mutants in mammalian cells. *Meth. Cell Biol.* 6:209-281.

Trowbridge, I. S., R. A. Newman, D. L. Domingo, and C. Sauvage. 1984. Transferrin receptors: structure and function. *Biochem. Pharmacol.* 33:925-932.

Ward, J. H., I. Jordon, J. P. Kushner, and J. Kaplan. 1984. Heme regulation of Hela cell transferrin receptor number. *J. Biol. Chem.* 259:13235-13240.

Wiley, H. S., and D. C. Cunningham. 1982. The endocytic rate constant. *J. Biol. Chem.* 257:4222-4229.

Wiley, H. S., and J. Kaplan. 1984. Epidermal growth factor rapidly induces a redistribution of transferrin receptor pools in human fibroblasts. *Proc. Natl. Acad. Sci. USA.* 81:7456-7460.

Yamashiro, D. J., B. Tycko, S. R. Fluss, and F. R. Maxfield. 1984. Segregation of transferrin to a mildly acidic (pH 6.5) para-Golgi compartment in the recycling pathway. *Cell.* 37:789-800.

Zerial, M., M. Suomalainan, M. Zanetti-Schnieder, C. Schneider, and H., Garoff. 1987. Phosphorylation of the human transferrin receptor by protein kinase C is not required for endocytosis and recycling in mouse 3T3 cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2661-2667.

Zoller, M. J., and M. Smith. 1982. Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any fragment of DNA. *Nucl. Acids Res.* 10:6487-6500.