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Association of phorbol ester-induced hyperphosphorylation and reversible regulation of transferrin membrane receptors in HL60 cells*

(transferrin receptor/cellular differentiation/tumor promoting agents)

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ABSTRACT **Phorbol diesters are tumor-promoting agents that cause differentiation of HL60 human leukemic cells and concomitantly alter surface transferrin-receptor expression [Rovera, G., Ferreo, D., Pagliardi, G. L., Vartikar, J., Pessano, S., Bottero, L., Abraham, S. & Lebman, D. (1982) *Ann. N.Y. Acad. Sci.* 397, 211–220]. Transferrin-receptor regulation is shown here to result from a rapid and reversible internalization process that is temporally associated with reversible increased phosphorylation (hyperphosphorylation) of the transferrin receptor. Such a reversible mechanism involving regulation of these surface proteins could result in the rapid generation of an early signal for HL60 cellular differentiation.**

Transferrin is a necessary component of virtually all serum-free tissue culture media (1), and for some cells it acts as a requisite growth factor (2, 3). Recently, the transferrin receptor has been identified and partially characterized (4, 5). It is a 180-kilodalton phosphorylated glycoprotein in its nonreduced homodimeric form (4-6). It is present in large amounts on the surface of rapidly proliferating cells of both malignant and normal origin (4), and it drastically diminishes when cells are induced to terminally differentiate (7-9).

HL60 cells are a human promyelocytic leukemic cell line that, when treated with tumor-promoting phorbol diesters, differentiate into functional monocyte-like cells (10). Treatment of HL60 cells with phorbol esters induces a rapid decline in surface transferrin-receptor numbers (7). This decline occurs relatively rapidly and it precedes both biochemical and morphological events associated with differentiation. Thus, the phorbol ester-induced loss of surface transferrin receptors in HL60 cells represents a unique system to study initial events related to induction of differentiation from the standpoint of changes occurring in a specific membrane receptor that has been universally associated with cellular proliferation.

We report here that, at early times, the decrease in surface-transferrin binding induced by phorbol dibutyrate (PDBu) is readily reversed by removing PDBu and is associated with receptor internalization and increased phosphorylation (hyperphosphorylation) of the transferrin receptor.

MATERIALS AND METHODS

Human transferrin was purchased from Sigma. Lactoperoxidase-linked Enzymobeads were obtained from Bio-Rad. Radioiodination of human ferrotransferrin was carried out using Enzymobeads and 100 μg of ferrotransferrin according to manufacturers' instructions. Ferrotransferrin was prepared using human transferrin that had been incubated with 4 mol of $FeCl_2$ per mol of transferrin for 4 hr at 4°C prior to iodination. Radioiodinated ferrotransferrin (^{125}I-transferrin) was separated using Sephadex G-25 gel chromatography. [Covalent coupling of ferrotransferrin to cyanogen bromide-activated Sepharose 4-B (Pharmacia) was carried out according to the manufacturers' recommendations.] PDBu and 4α-phorbol were purchased from Sigma, and a 50 μM stock solution in dimethylsulfoxide was prepared and stored at −20°C. A 1:500 dilution of this stock solution was carried out to yield a 100 nM final concentration of those agents.

Cell Culture. HL60 cells were grown in a spinner flask at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum/10 mM Hepes buffer, pH 7.2/penicillin/streptomycin. Cells grown to logarithmic phase were used for all experiments unless stated otherwise.

Equilibrium Binding of ^{125}I-Transferrin to HL60 Cells. To detect surface binding of ^{125}I-transferrin by whole cells, cells were grown to logarithmic phase and were harvested by centrifugation for 5 min at 1000 rpm and washed once with RPMI 1640 medium at 37°C, containing fetal bovine serum and 10 mM Hepes (pH 7.2). Cells were resuspended in this medium at the same density. After additions were made to the cells, incubation at 37°C was continued for 1 hr. After incubation, cells were harvested by centrifugation as described above and washed twice with RPMI 1640 medium containing 20 mM Hepes buffer (pH 7.2) at 4°C. Cells were suspended at 10×10^6 cells per ml in the same buffer, which now contained turkey ovalbumin at 0.5 mg/ml (binding buffer). Cells in binding buffer were equilibrated for 15 min at 4°C prior to addition of ^{125}I-transferrin. Increasing concentrations of ^{125}I-transferrin were made to cells and binding was carried out for 60 min, which corresponded to the previously determined equilibrium binding time (data not shown). After incubation at 4°C, triplicate 200-μl aliquots of cells (2×10^6 cells) were pipeted into 400-μl Microfuge tubes (Markson, Phoenix, AZ) and centrifuged in a Beckman 152 Microfuge for 90 sec at 4°C. After complete aspiration of the supernatant, the cell pellet was excised and placed into an appropriate tube for gamma counting. Results are corrected for nonspecific binding obtained in the presence of a 1000-fold excess of unlabeled ferrotransferrin and represent specific binding of ^{125}I-ferrotransferrin (i.e., total binding minus nonspecific binding equals specific binding).

To detect total ^{125}I-transferrin-receptor binding (surface plus internal), cells were treated with PDBu or 4α-phorbol as described in the legend to Table 1. Cells were divided and whole-cell-equilibrium binding (surface) was determined as described on half of the cells, and total binding on permeabilized cells (extract) (11) with modifications as described below was determined on the remainder of the cells. Intact whole cells were washed once at 4°C as described. Cells

Abbreviations: PDBu, phorbol dibutyrate; ^{125}I-transferrin, ^{125}I-labeled ferrotransferrin.

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were then permeabilized in 50 mM Tris·HCl, pH 7.2/150 mM NaCl/1.5 mM $MgCl_2$/1 mM phenylmethylsulfonyl fluoride/turkey ovalbumin (0.5 mg/ml) (Sigma)/0.1% Triton X-100 at 4°C. The volume of extraction was the same as that into which whole cells had been suspended for equilibrium-binding studies. Cells were allowed to incubate at 4°C for 25 min with frequent vigorous Vortex mixing. This cellular extract was then used as the source of total receptor material. The same volume of intact cells and cellular extract (i.e., 200 μl) was used for whole cell ^{125}I-transferrin equilibrium binding (as described previously) and for total ^{125}I-transferrin binding, as determined in the following manner. To 200-μl aliquots of cell extract was added 21 nM final concentration of ^{125}I-transferrin. Incubation was carried out for 60 min at 4°C, at which time was added 300 μl of 100 mM citrate buffer (pH 5.5) containing 150 mM NaCl/ovalbumin (0.5 mg/ml)/bovine IgG (1 mg/ml). After Vortex mixing, incubation was continued for 10 min at 4°C. Then, 1 ml of 50 mM citrate buffer (pH 5.5) containing 150 mM NaCl/ovalbumin (0.5 mg/ml)/15% polyethylene glycol 6000 was added. After Vortex mixing, the suspension was incubated at 4°C for an additional 15 min and then centrifuged at 3500 rpm for 20 min at 4°C in a Sorvall RC-3. After centrifugation, supernatant was aspirated and the precipitated pellet containing ^{125}I-transferrin bound to its specific receptor was excised from the tube and counted in a gamma counter. Results are expressed as specific cpm bound per 10^6 cells (surface) or per 10^6 cell equivalents (extract). Specific cpm bound represents the difference between total cpm of ^{125}I-transferrin bound minus cpm of ^{125}I-transferrin bound in the presence of a 1000-fold excess of unlabeled ferrotransferrin.

Isolation of Phosphorylated Transferrin Receptors by Affinity Chromatography from Solubilized Whole Cells. Cells were washed twice in phosphate-free RPMI 1640 medium containing 20 mM Hepes buffer at pH 7.2, and resuspended in this same buffer at 0.5–1 × 10^8 cells per ml. [^{32}P]Orthophosphate (1.5 mCi/ml; 1 Ci = 37 GBq) was added and the cells were incubated for 3 hr at 37°C with gentle tumbling. At this point, additions were made and incubation at 37°C continued for various times up to 60 min. At specified times, 1 ml of incubating cells was placed into 14 ml of 50 mM Tris buffer (pH 7.2) containing 150 mM NaCl at 4°C and centrifuged for 5 min at 1000 rpm. Two additional washings of these cells under the same conditions were carried out. The resulting cell pellet was suspended by Vortex mixing into 1.2 ml of the same buffer containing 1.5% Triton X-100/1.5 mM $MgCl_2$/20 mM sodium fluoride/0.1 mM sodium vanadate/bacitracin (1 mg/ml)/1 mM phenylmethylsulfonyl fluoride. Resulting cell lysates were vigorously mixed in a Vortex at ≈5-min intervals for 25 min. Then, cell lysates were centrifuged for 20 min at 3500 rpm at 4°C. The resulting supernatant was used as the starting material for affinity chromatography isolation of the soluble transferrin-membrane receptor. To 1 ml of this soluble membrane preparation (contained in a 1.5-ml Eppendorf tube) was added 100 μl of Sepharose 4-B-cyanogen-transferrin affinity gel. Incubation was carried out with gentle tumbling for 16 hr at 4°C. The transferrin-affinity gel containing bound phosphorylated transferrin receptor was then washed 3 times with 50 mM Tris buffer (pH 7.2) containing 150 mM NaCl/0.1% Triton X-100/bacitracin (1 mg/ml). Washing consisted of sequential additions of 1 ml of buffer to the affinity gel pellet. This was followed by a 30-sec centrifugation on a Beckman 152 Microfuge. The supernatant was aspirated and discarded. After three such washings, affinity-bound transferrin membrane receptors were eluted from the gel by incubating the washed affinity gel pellet with 125 M Tris buffer (pH 6.8) containing 3% $NaDodSO_4$. Incubation was carried out for 5 min at room temperature followed by a 5-min incubation in a boiling water bath. To the resultant eluate was added 25 μl of 5 times concentrated $NaDodSO_4$ sample buffer containing 400 mM dithiothreitol/0.1% bromophenol blue dye.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis and Autoradiography. $NaDodSO_4$/polyacrylamide gel electrophoresis was performed under reducing conditions by the procedure of Laemmli (12) with separating gel of 8% acrylamide and stacking gel of 3% acrylamide. Iodinated transferrin receptor used as a radiolabeled standard was prepared by iodinating HL60 whole cells using the lactoperoxidase method. The ^{125}I-transferrin receptor was then isolated using the affinity procedure described here.

After electrophoretic separation the polyacrylamide gel was dried onto Whatman paper. Subsequent autoradiography using Kodak X-Omat AR film was performed.

RESULTS

Reversible Down-Regulation of Surface-Transferrin Receptors. Incubation of HL60 cells with 100 nM PDBu for 1 hr at 37°C causes a 50% loss (i.e., down-regulation) in detectable ^{125}I-transferrin surface binding (Fig. 1). This is due to a decreased number of accessible receptors (1.2 vs. 0.6 × 10^5 receptors per cell for control and PDBu-treated cells, respectively) with no significant change in the dissociation constant (K_d) for transferrin binding (3.5 and 4.8 × 10^{-9} M for control and PDBu-treated cells, respectively). Incubation of cells with PDBu for longer times causes further down-regulation of transferrin surface receptors so that after 12 hr, surface-transferrin binding is essentially not detectable (data not shown). By 12 hr after PDBu addition, a significant proportion of HL60 cells have become adherent, which corresponds to morphological and functional differentiation changes that characterize macrophages (13).

As shown in Fig. 2, down-regulation of transferrin receptors is specific for active, tumor-promoting diesters of phorbol. The non-tumor-promoting 4α-phorbol has no such effect. Fig. 2 also shows that down-regulation is rapidly reversible when PDBu is removed. Recovery of transferrin receptors is independent of the synthesis of new receptors, because it occurs when protein synthesis is blocked by a 2-hr incubation with a high concentration of cycloheximide.

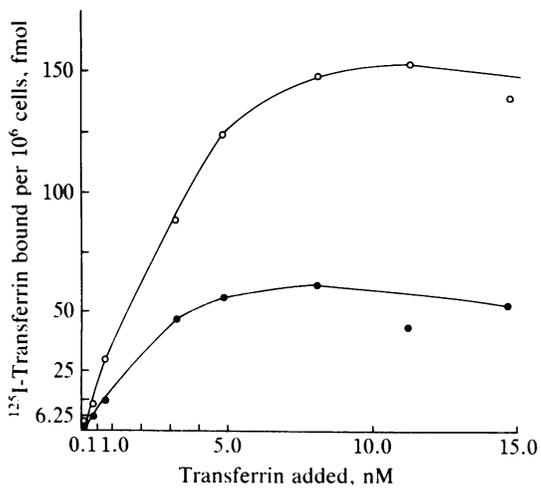

FIG. 1. Equilibrium binding of ^{125}I-transferrin to HL60 cells. To whole cells was added either 100 nM PDBu or 100 nM 4α-phorbol (control). After a 60-min incubation at 37°C, cells were washed and used for equilibrium binding studies. For control cells (○) the dissociation constant (K_d) is 3.5 × 10^{-9} M and the total number of transferrin receptors per cell is 1.2 × 10^5. For cells incubated with PDBu (●) results reveal that the K_d is 4.8 × 10^{-9} M and the total number of transferrin receptors per cell is 0.6 × 10^5. When 100 nM PDBu is added to control cells at 4°C, no inhibition of ^{125}I-transferrin binding is observed. Cycloheximide at 10 μg/ml was added to whole cells 1 hr before addition of PDBu in order to inhibit protein synthesis.

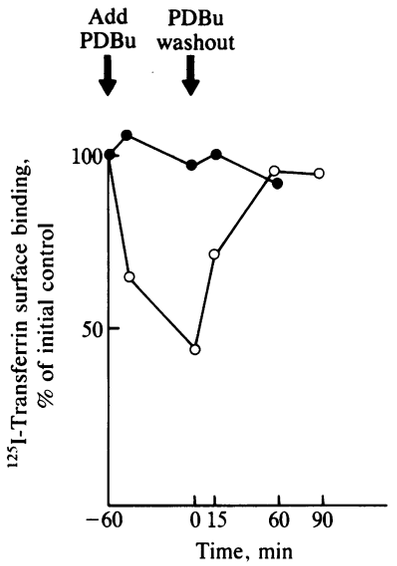

FIG. 2. Reversible loss of ^{125}I-transferrin surface binding by HL60 cells incubated with PDBu. Logarithmic phase HL60 cells were washed once and resuspended; 100 nM PDBu (○) or 4α-phorbol (●) was added to cells incubating at 37°C (−60 min). Incubation in the presence of these agents was carried out for up to 60 min. The cells were washed twice with RPMI 1640 medium at 4°C containing 10% fetal bovine serum and 10 mM Hepes, pH 7.2, and resuspended at the same density in wash medium that had been warmed to 37°C, and contained cycloheximide at 10 μg/ml. Incubation at 37°C was continued for up to 90 min. At various times during the first and second incubations, cells were harvested and washed, and equilibrium binding studies with saturating concentrations of ^{125}I-transferrin (21 nM) were carried out. Cycloheximide at 10 μg per ml of medium was added 2 hr prior to additions and was present throughout, except during equilibrium binding studies at 4°C.

Decrease in ^{125}I-Transferrin Surface Binding After Incubation with PDBu Is Associated with Surface-Receptor Internalization. Since the decrease in transferrin receptor numbers that occurs when cells are incubated with PDBu, as well as their recovery when PDBu is removed, occurs in the absence of new protein synthesis, a possible mechanism for these processes is the reversible translocation of receptor from the cell surface. To evaluate this hypothesis, the number of cell surface receptors (measured by transferrin binding to intact cells at 4°C) was compared with the number of total cellular receptors (measured by transferrin binding after the cells are disrupted with Triton X-100). The results are presented in Table 1. When cells are treated with PDBu, there is more than a 50% decrease in cell-surface transferrin binding compared to control cells or cells treated with the non-tumor promotor 4α-phorbol. However, incubation with PDBu does not significantly change the total number of cellular transferrin receptors. Thus, in the basal state, ≈95% of the transferrin receptors are accessible on the cell surface. After 1-hr treatment with PDBu, there is a translocation of receptors from the cell surface so that ≈50% remain at the cell surface, while the remainder have entered an internal pool; internalized transferrin receptors are accessible after detergent disruption of whole cells with Triton X-100. Notably, this translocation of surface receptors occurs in the absence of exogenous transferrin.

Table 1. Recovery of PDBu-induced loss of ^{125}I-transferrin surface binding by whole cells after detergent disruption of whole cells

Addition	cpm per 10^6 cells or cell equivalents		Distribution of receptors after incubation	
	Surface	Extract	% ext.	% int.
None	3078	3146	98	2
4α-Phorbol	3209	3361	95	5
PDBu	1512	3431	44	56

HL60 cells were treated with no additions, 100 nM 4α-phorbol, or 100 nM PDBu. After incubation with these agents, each sample was divided in half and measurement of whole-cell equilibrium binding (surface) as well as total soluble transferrin receptors (extract) was carried out. % ext. represents calculations for percent of whole-cell surface receptors. % int. represents percent of whole-cell internal receptors calculated from the difference in specific cpm bound between extract and surface results.

Rapid Hyperphosphorylation of Transferrin Receptors on Incubation of Cells with PDBu. Intact HL60 cells were incubated with [^{32}P]orthophosphate for 3 hr to label their endogenous pool of ATP, and then for an additional period of time in the presence of PDBu or 4α-phorbol. These cells were then solubilized, and the transferrin receptor was isolated by affinity chromatography and the proteins were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 3*A*). PDBu (100 nM) causes a rapid increase in phosphorylation of the receptor, which begins within 5 min. This hyperphosphorylation appears to be maximal by about 15 min (Fig. 3*B*). By contrast, there is no change in the apparent quantitative phosphorylation of the receptor isolated from cells incubated with the inactive 4α-phorbol compound.

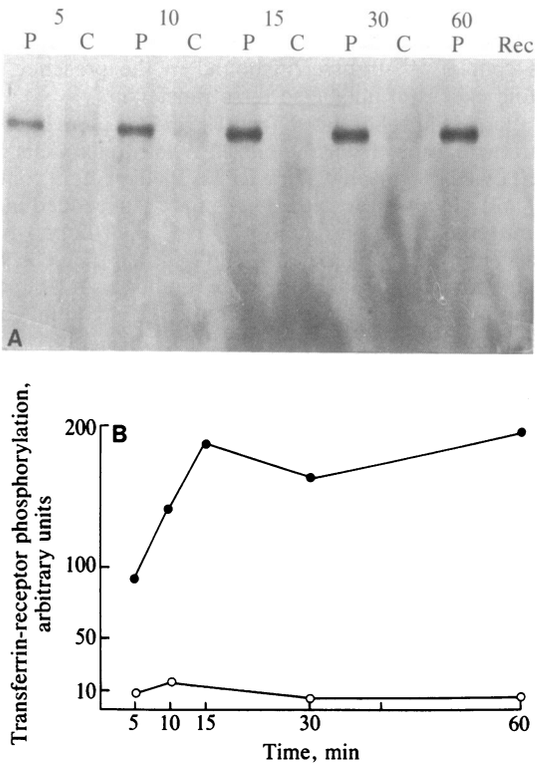

FIG. 3. Time course for hyperphosphorylation of the transferrin membrane receptor of HL60 cells incubated with PDBu. Cells were incubated with [^{32}P]orthophosphate, and then either 100 nM PDBu (P) or 4α-phorbol (C) was added and incubation continued. At specified times up to 60 min whole cells were collected and phosphorylated transferrin receptor was isolated. The resulting autoradiogram (*A*) was exposed for 16 hr for optimal development of the film. Each time point (in minutes) contains results for transferrin-receptor phosphorylation in the presence of PDBu or 4α-phorbol. The lane labeled Rec represents electrophoretic migration of iodinated transferrin receptor used as the radiolabeled standard. (*B*) Plot of arbitrary units of transferrin-receptor phosphorylation obtained by laser densitometric scanning of each lane of the autoradiogram. ●, Receptors isolated from cells incubated with PDBu; ○, cells incubated with 4α-phorbol (control).

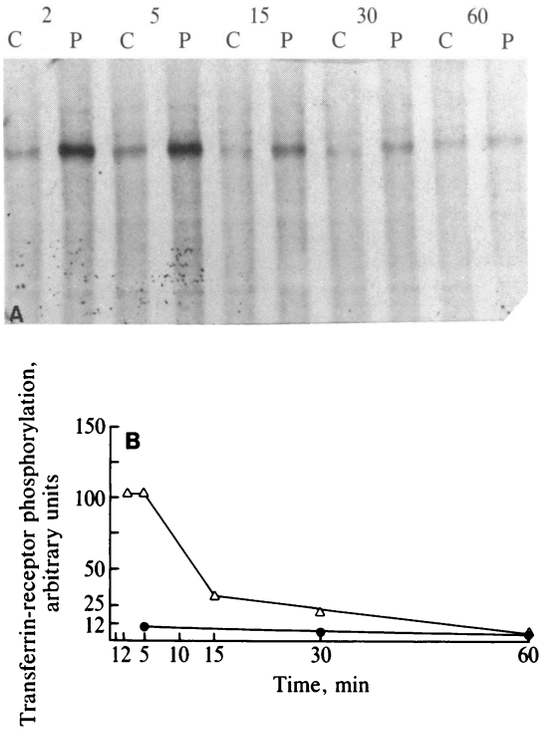

FIG. 4. Time course for dephosphorylation of the transferrin membrane receptor of HL60 whole cells after removal of PDBu from the incubation medium. Cells were incubated with [^{32}P]orthophosphate and either PDBu (P) or 4α-phorbol (C). After incubation for 30 min with either PDBu or 4α-phorbol, cells were washed and resuspended into the same initial volume of RPMI 1640 medium containing 2% fetal bovine serum/penicillin/streptomycin/20 mM Hepes, pH 7.2/1 mM ATP. Incubation at 37°C was carried out for various times (in minutes) up to 60 min. At specified times, 1-ml aliquots of both PDBu- and 4α-phorbol-treated cells were removed into 14 ml of wash buffer and treated. (*A*) Optimally exposed autoradiogram of phosphorylated transferrin receptors isolated at various times after removal of phorbol additions. (*B*) Plot of the arbitrary units of transferrin-receptor phosphorylation obtained by laser densitometric scanning of each lane of the autoradiogram, which contained isolated phosphorylated transferrin receptors. △, Receptors isolated after treatment with PDBu; ●, similar incubations with the 4α-phorbol (control) compound.

PDBu-induced phosphorylation of the receptor is apparently maximal prior to achievement of maximal down-regulation of surface receptors (compare Fig. 3*B* and Fig. 2). This suggests that hyperphosphorylation of the receptor may precede the PDBu-induced internal translocation process that results in receptor down-regulation.

Rapid Dephosphorylation of Transferrin Receptors After Removal of PDBu from the Incubation Medium. The state of increased phosphorylation of the transferrin receptor induced by PDBu is rapidly reversed to basal receptor phosphorylation levels when PDBu is removed (Fig. 4). Dephosphorylation begins after a 5-min lag period (Fig. 4*B*), which probably represents initial reequilibration of residual cell-bound PDBu. Subsequent dephosphorylation proceeds rapidly from this point; the transferrin receptor reaches the basal state of phosphorylation by about 30 min after removal of PDBu (Fig. 4*B*). No change in the status of phosphorylation of the receptor is observed in cells incubated with 4α-phorbol.

In addition (compare Fig. 4 with Fig. 2), the time course for maximal decay of the phosphorylated state of the receptor is faster than that of maximal recovery of surface-transferrin binding. This suggests the occurrence of rapid receptor regulation through a phosphorylation mechanism.

It has been further shown that PDBu-induced receptor hyperphosphorylation is associated with increased phosphorylation on serine and threonine amino acid residues, with no demonstrable *in vivo* tyrosine phosphorylation noted (results not shown).

DISCUSSION

These results confirm the findings of Rovera *et al.* (7) that phorbol esters rapidly decrease the number of receptors on the surface of HL60 cells. In addition, they show that this decrease is not due to catabolism of the receptors, since recovery from down-regulation is not inhibited when new protein synthesis is blocked with cycloheximide. They also show that the cell surface receptors that disappear can be quantitatively recovered by disrupting cells with Triton X-100. In view of these findings, the most likely mechanism for down-regulation is the reversible translocation of receptors to some intracellular or inaccessible pool. When transferrin binds to its receptor on the cell surface, both the receptor and bound ligand are internalized (11, 14–16). The complex rapidly comes into contact with an acidic compartment where iron dissociates, and the receptor with apotransferrin still bound is rapidly recycled to the cell surface (11, 14, 15). Whether phorbol esters induce internalization via a similar pathway and, if so, whether phorbol esters specifically lead to a block in the recycling of the sequestered receptors are questions for further investigation.

However, since transferrin-induced hyperphosphorylation of its own receptor apparently does not occur (data not shown), the possible role of enhanced receptor phosphorylation in internalization or sequestration induced by phorbol esters would appear to be unrelated to the usual cycling mechanism of the ferrotransferrin–receptor complex, which is initiated by ligand binding (11, 14–16). Furthermore, transferrin also does not inhibit PDBu-induced transferrin-receptor hyperphosphorylation (data not shown). These data suggest that direct phosphorylation of the receptor does not alter affinity for transferrin binding because full pretreatment values for ^{125}I-transferrin binding are recovered after PDBu-induced receptor down-regulation.

The transferrin receptor is known to be a phosphoprotein (6). In this work, it is shown that PDBu reversibly enhances the extent of phosphorylation of the receptor. Since phorbol esters specifically activate protein kinase C and cause its redistribution from the cytosol to the cell membrane (17, 18), it is possible that the increased phosphorylation of the transferrin receptor is a direct effect of protein kinase C. Consistent with this, hyperphosphorylation of the transferrin receptor occurs on serine and threonine residues, which are the known targets for protein kinase C (19). In this regard, it is interesting that when cells are incubated with phorbol esters, there is enhanced phosphorylation of the insulin and somatomedin-C receptors (20), and apparently also the epidermal growth factor receptor (21), as reported in a recent abstract. In addition both insulin (22, 23) and epidermal growth factor (24) binding to their receptors is decreased after phorbol ester treatment. However, decreased insulin and epidermal growth factor binding are apparently due to a major decrease in receptor affinity (22–24), while with transferrin receptors, a decreased number of surface receptors is observed.

The role of PDBu-enhanced transferrin receptor phosphorylation is not clear. Recently, receptor phosphorylation has received much attention as a possible mechanism for transmembrane signaling (25–27). Several mitogenic hormones including epidermal growth factor (28), platelet-derived growth factor (29, 30), insulin (31, 32), and somatomedin C appear to induce a tyrosine-specific protein kinase that is stimulated by hormone binding and is capable of autophosphorylation of these hormone receptors. In contrast to this

phenomenon, transferrin binding to its own receptor does not alter the extent of basal receptor phosphorylation, and phosphotyrosine is apparently not found in the transferrin receptor.

While receptor-related tyrosine phosphorylation may be associated with a regulatory role for cellular growth in the case of certain mitogenic hormones (25, 26, 28, 33), any such putative role for transferrin does not appear to result from changes in transferrin-receptor tyrosine phosphorylation. However, it is possible, albeit speculative, that the effects of phorbol esters on inducing cellular differentiation might result from initial changes in the status of phosphorylation of the transferrin receptor or some other surface protein(s). Such effects are likely to be independent of any ligand-induced changes that may occur on these receptors. Thus, differential states of receptor phosphorylation [with respect to amino acid residues and peptide region(s) so involved] may occur depending on the stimulus (i.e., ligand, tumor promotor, etc.). Such alterations may then be involved in subsequent cellular regulatory processes, at least in some cases, leading to altered states of differentiation. It follows from this scheme that a defect in this pathway could lead to uncontrolled cell growth or inability to progress from a less-differentiated cellular state.

A likely specific role for PDBu-enhanced phosphorylation of the transferrin receptor is as a signal for receptor internalization and intracellular sequestration. The kinetics of receptor hyperphosphorylation are consistent with this. In the presence of PDBu, the time course of phosphorylation precedes the net loss of surface receptors, while after removal of PDBu, reversal of phosphorylation again precedes the full reappearance of receptors at the cell surface. This entire process presumably requires rapid action by both kinase and phosphatase activities; the former is associated with phosphorylation and receptor down-regulation, while the latter is associated with rapid receptor dephosphorylation and up-regulation.

The possible role of PDBu-induced changes in transferrin receptors in triggering cellular differentiation or other biological phenomena is at present not clear.

Note Added in Proof. Two papers have appeared reporting phorbol ester (34) and protein kinase C (35) stimulation of phosphorylation of the epidermal growth factor receptor.

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1. Barnes, D. & Sato, G. (1980) *Anal. Biochem.* **102,** 255–270.
2. Ekblom, P., Thesleff, I., Saxén, L., Miettinens, A. & Timpl, R. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 2651–2655.
3. Neckers, L. M. & Cossman, J. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 3494–3498.
4. Sutherland, R., Delia, D., Schneider, C., Newman, R., Kemshead, J. & Greaves, M. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 4515–4519.
5. Newman, R., Schneider, C. S., Sutherland, R., Vodinelich, L. & Greaves, M. (1982) *Trends Biochem. Sci.* **7,** 397–400.
6. Schneider, C., Sutherland, R., Newman, R. & Greaves, M. (1982) *J. Biol. Chem.* **257,** 8516–8522.
7. Rovera, G., Ferreo, D., Pagliardi, G. L., Vartikar, J., Pessano, S., Bottero, L., Abraham, S. & Lebman, D. (1982) *Ann. N.Y. Acad. Sci.* **397,** 211–220.
8. Pan, B., Blotstein, R. & Johnstone, R. M. (1983) *Biochem. J.* **210,** 37–47.
9. Yeh, C. G., Papamichael, M. & Faulk, W. P. (1982) *Exp. Cell Res.* **138,** 429–433.
10. Abraham, J. & Rovera, G. (1980) *Mol. Cell. Biochem.* **31,** 165–175.
11. Klausner, R. D., Renswoude, J. V., Ashwell, G., Kempf, C., Schechter, A. N., Dean, A. & Bridges, K. R. (1983) *J. Biol. Chem.* **58,** 4715–4724.
12. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
13. Cooper, R. A., Brunwald, A. D. & Kuo, A. L. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 2865–2869.
14. Dautry-Varsat, A., Ciechanover, A. & Lodish, H. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 2258–2262.
15. Brown, M. S., Anderson, R. G. W. & Goldstein, J. L. (1983) *Cell* **32,** 663–667.
16. Karin, M. & Mintz, B. (1981) *J. Biol. Chem.* **256,** 3245–3252.
17. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. & Nishizuka, Y. (1982) *J. Biol. Chem.* **257,** 7847–7851.
18. Kraft, A. S. & Anderson, W. B. (1983) *Nature (London)* **301,** 621–623.
19. Yasushi, I., Yoshimi, T., Kikkawa, U. & Nishizuka, Y. (1980) *Biochem. Biophys. Res. Commun.* **96,** 180–187.
20. Jacobs, S. J., Sahyoun, N., Saltiel, A. & Cuatrecasas, P. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 6211–6213.
21. Iwashita, S. & Fox, C. F. (1983) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **42,** 1902 (abstr.).
22. Thomopoulos, P., Testa, Y., Gourdin, M., Hervy, C., Titeux, M. & Vainchenker, W. (1982) *Eur. J. Biochem.* **129,** 389–393.
23. Grunberger, G. & Gorden, P. (1982) *Am. J. Physiol.* **243,** E319–E324.
24. Shoyab, M., DeLarco, J. E. & Todaro, G. J. (1979) *Nature (London)* **279,** 387–391.
25. Rosen, O. M., Herrera, R., Olowe, Y. Petruzzelli, L. M. & Cobb, M. H. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 3237–3240.
26. Sefton, B. M., Hunter, T., Beemon, K. & Eckhart, W. (1980) *Cell* **20,** 807–816.
27. Kolata, G. (1982) *Science* **219,** 377–378.
28. Cohen, S., Carpenter, G. & King, L. (1980) *J. Biol. Chem.* **255,** 4834–4842.
29. Cooper, J. A., Bowen-Pope, D. F., Raines, E., Ross, R. & Hunter, T. (1982) *Cell* **31,** 263–273.
30. Denton, R. M., Brownsey, R. W. & Belsham, G. J. (1981) *Diabetologia* **21,** 347–362.
31. Zick, Y., Whittaker, J. & Roth, J. (1983) *J. Biol. Chem.* **258,** 3431–3434.
32. Zick, Y., Kasuga, M., Kahn, C. R. & Roth, J. (1983) *J. Biol. Chem.* **258,** 75–80.
33. Ek, B., Westermark, B., Wasteson, A. & Heldin, C. (1982) *Nature (London)* **295,** 419–420.
34. Iwashita, S. & Fox, C. F. (1984) *J. Biol. Chem.* **259,** 2559–2567.
35. Cochet, C., Gill, G. N., Meisenhelder, J., Cooper, J. & Hunter, T. (1984) *J. Biol. Chem.* **259,** 2553–2558.