Regulation of Transferrin Receptor Cycling by Protein Kinase C Is Independent of Receptor Phosphorylation at Serine 24 in Swiss 3T3 Fibroblasts*

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Transferrin is a serum protein that binds iron and is an essential requirement for the growth of cultured cells (1). The uptake of iron into cells is mediated by specific cell surface receptors for diferric transferrin that cycle between the plasma membrane and endosomal membranes (reviewed in Ref. 2). Activation of the Ca\(^{2+}\)/phospholipid-dependent protein kinase (protein kinase C) \(^*\) by treatment of cells with phorbol diester \((\Delta 6, \Delta 11\)-di-O-tetradecanoylphorbol-13-acetate) for the wild-type receptor. We conclude that the regulation of transferrin receptor cycling by protein kinase C is independent of receptor phosphorylation at serine 24 in Swiss 3T3 fibroblasts.

Treatment of Swiss 3T3 fibroblasts with tumor-promoting phorbol diester or with platelet-derived growth factor caused the phosphorylation of the transferrin receptor by protein kinase C \((\text{Ca}^{2+}/\text{phospholipid-dependent enzyme})\) at serine 24 and increased the cell surface expression of the transferrin receptor. The hypothesis that the regulation of transferrin receptor cycling by protein kinase C is causally related to the phosphorylation of the receptor at serine 24 was critically tested. Site-directed mutagenesis of the human transferrin receptor cDNA was used to substitute serine 24 with threonine or alanine residues in order to create phosphorylation defective receptors. Wild-type and mutated transferrin receptors were expressed in Swiss 3T3 fibroblasts using the retrovirus vector pZipNeoSV (X). These receptors were functionally active and caused the receptor-mediated endocytosis of diferric transferrin. Incubation of the fibroblasts with phorbol diester caused the phosphorylation of the wild-type (Ser-24) human transferrin receptor, but this treatment did not result in the phosphorylation of the mutated (Ala-24 and Thr-24) receptors. The cycling of the phosphorylation defective receptors was regulated by phorbol diester and platelet-derived growth factor in a manner similar to that observed for the wild-type receptor. We conclude that the regulation of transferrin receptor cycling by protein kinase C is independent of receptor phosphorylation at serine 24 in Swiss 3T3 fibroblasts.

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The abbreviations used are: protein kinase C, \(\text{Ca}^{2+}/\text{phospholipid-dependent protein kinase}\); EGF, epidermal growth factor; EGTA, \(\text{ethylenbis(oxyethylenenitrilo)}\)tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PDGF, platelet-derived growth factor; PMA, \(\Delta 6/\Delta 11\)-phorbol 12-myristate 13-acetate; kb, kilobase pairs.

such as PDGF (9) which stimulate the activity of protein kinase C (10-14).

The mechanism by which protein kinase C regulates the cycling of the transferrin receptor is not understood. May et al. (4, 15, 16) have proposed that phosphorylation of the transferrin receptor by protein kinase C regulates cycling. The purpose of the experiments described here was to critically test this hypothesis. The approach that we took was to examine the regulation of the cycling of transferrin receptors that are defective as protein kinase C substrates. The site of transferrin receptor phosphorylation by protein kinase C has been identified as serine 24 (17). By site-directed mutagenesis we substituted serine 24 with a threonine and an alanine residue and expressed these mutant receptors in Swiss 3T3 cells using a retrovirus vector. The cycling of the phosphorylation defective receptors was regulated by PMA in a manner similar to that observed for the wild-type receptor. We conclude that the regulation of transferrin receptor cycling by protein kinase C is independent of receptor phosphorylation at serine 24 in Swiss 3T3 fibroblasts.

**EXPERIMENTAL PROCEDURES**

*Materials—\([\text{32P}]\)Phosphate was obtained from Du Pont-New England Nuclear. \(\text{FeCl}_3\), \(\text{Na}_{125}\text{I}\), nucleotides, \([\text{125I}]\)labeled mouse IgG, \([\text{125I}]\)labeled anti-rat IgG, and \([\text{125I}]\)methionine were from Amer sham Corp. Restriction enzymes were from New England Biolabs. Poly-nucleotide kinase and Klenow were from Pharmacia and United States Biochemical Corp., respectively. Dideoxynucleotides were from Boehringer Mannheim. Phorbol diesters and protein A-Sepharose CL-4B were from Sigma. The synthetic peptide Lys-Arg-Thr-Leu-Arg-Arg was obtained from Peninsula Laboratories (Belmont, CA). Epidermal growth factor was purified (18, 19) and iodinated (20) as described. Platelet-derived growth factor (porcine) was from Biopro cessing Ltd. G418 was obtained from Gibco. Transferrin was from Behring Diagnostics and was further purified by gel filtration chromatography before use. Fluorescein-conjugated antibodies were from Cappel. Diferric transferrin, \([\text{59Fe}]\)diferric transferrin, and diferric \(\text{ACCGGGTATA}-3')\) or alanine (5'-AGWGAACCGGGTm-3').

**Cell Culture—**Swiss 3T3 cells were obtained from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% calf serum. Psi-2 cells were obtained from Dr. R. Mulligan (Whitehead Institute). WI-38 human fetal lung fibroblasts were obtained from the American Type Culture Collection and maintained in modified Eagle’s medium supplemented with 5% fetal calf serum. Hybridoma cells OKT9, R17217, and R17208 were obtained from the American Type Culture Collection.

The mechanism by which protein kinase C regulates the cycling of the transferrin receptor is not understood. May et al. (4, 15, 16) have proposed that phosphorylation of the transferrin receptor by protein kinase C regulates cycling. The purpose of the experiments described here was to critically test this hypothesis. The approach that we took was to examine the regulation of the cycling of transferrin receptors that are defective as protein kinase C substrates. The site of transferrin receptor phosphorylation by protein kinase C has been identified as serine 24 (17). By site-directed mutagenesis we substituted serine 24 with a threonine and an alanine residue and expressed these mutant receptors in Swiss 3T3 cells using a retrovirus vector. The cycling of the phosphorylation defective receptors was regulated by PMA in a manner similar to that observed for the wild-type receptor. We conclude that the regulation of transferrin receptor cycling by protein kinase C is independent of receptor phosphorylation at serine 24 in Swiss 3T3 fibroblasts.

*Site-directed Mutagenesis—* The full-length 4.9-kb cDNA for the human transferrin receptor in the plasmid pcDTR1 was provided by Dr. F. H. Ruddle (Yale University) (21). A BamHI-HindIII 0.9-kb fragment isolated from pcDTR1 (which contains the 5'-untranslated region and the coding region for the amino-terminal cytoplasmic domain) was subcloned into M13mp8. Site-directed mutagenesis of serine 24 (AGC) was carried out according to Zoller and Smith (22) using 17-mer oligonucleotides coding for threonine (5'-AGGGTGAGCGGTTATA-3') or alanine (5'-AGGCACCGGGTATA-3'). Mutants were selected by ligation at increasing temperatures in 6 x
Transferrin Receptor Phosphorylation by Protein Kinase C

N-acetyl-glucosamine, pH 7.5 (23), and confirmed by sequencing the entire 0.9-kb fragment with [35S]dATP and ddNTPs (24). The native BamHI-HindIII 0.9-kb fragment as well as the alanine and threonine mutants at amino acid 24 were isolated and ligated to a HindIII-BglII 1.9-kb fragment of pcTTR1 that contains the coding region for the carboxyterminal domain of the transferrin receptor in both orientations (25). After digestion with BamHI and BglII, a 2.8-kb cDNA containing the coding region and portions of the untranslated regions was isolated by agarose gel electrophoresis and cloned into the BamHI site of the retrovirus vector pZIPNeoSV(X) (25) in both orientations using standard techniques (26). The plasmids obtained pZTSer-24, pZTAla-24, and pZTThr-24 were used for the expression of wild-type (Ser-24) and mutated human transferrin receptors in murine cells.

Expression of the Transferrin Receptor cDNA in Swiss 3T3 Cells—The plasmid constructs pZTThr-24, pZTRAla-24, and pZTThr-24 were transfected into FCS-2 cells using the CaPO4 method (25). Stable colonies resistant to G418 (1 mg/ml) were isolated by agorse gel electrophoresis and cloned into the BamHI site of the retrovirus vector pZIPNeoSV(X) (25) in both orientations using standard techniques (26). The plasmids obtained pZTThr-24, pZTAla-24, and pZTThr-24 were used for the expression of wild-type (Ser-24) and mutated human transferrin receptors in murine cells.

Phosphopeptide Mapping—Transferrin receptors were isolated from [32P]phosphate-labeled cells by immunoprecipitation and polyacrylamide gel electrophoresis. The receptors were digested with trypsin and the [32P]phosphopeptides obtained were resolved by high pressure liquid chromatography as described (17). A Vydac C8 column equilibrated with 0.1% trifluoroacetic acid was employed. After injection of the sample, the column was washed for 5 min, and the peptides were eluted with a linear gradient of acetonitrile (0-60%) over 60 min. The flow rate was 1 ml/min. Fractions eluted from the column were collected, and peptides were detected by Cerenkov counting.

Measurement of Protein Kinase C Function and Activity—The specific binding of 8 nM [3H]PDBU to cell monolayers was measured as described (30). The regulation of the EGF receptor was investigated by measuring the specific binding of [125I]EGF to cell monolayers as described (30). Protein kinase C activity was measured using a synthetic peptide substrate assay.

Analysis of Transferrin Binding and Uptake—The binding of [125I]transferrin to cell surface receptors was performed as described previously (9). The uptake of [125I]transferrin by cells was measured at 37 °C (9). The rate of release of [125I]transferrin from cell monolayers was measured as described by Wiley and Kaplan (32).

Indirect Immunofluorescence—Cells were rapidly transferred from medium at 37 °C to 3% (w/v) formaldehyde, 120 mM NaCl, 6 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 25 mM Hepes (pH 7.4) at 22 °C. After 20 min the cells were washed and then incubated with 50 mM NH4Cl in Hepes-buffered saline for 10 min. Subsequently, the cells were incubated for 3 min with 0.2% Triton X-100 in Hepes-buffered saline to expose intracellular antigenic sites. The cells were washed and then incubated with 20 μg/ml anti-human transferrin receptor monoclonal antibody OKT9 for 40 min at 22 °C. The cells were then washed and incubated with fluorescein-conjugated goat anti-mouse IgG antibody (1:300) for 40 min. After this incubation the cells were washed and examined by fluorescence microscopy. In other experiments the monoclonal antibody R17217 was employed and fluorescein-conjugated goat anti-rat IgG antibody was used to visualize the murine transferrin receptors.

RESULTS

Expression of the Human Transferrin Receptor in Swiss 3T3 Fibroblasts—Expression of the human transferrin receptor in Swiss 3T3 fibroblast clones was investigated by immunoprecipitation of cells metabolically labeled with [35S]methionine using a monoclonal antibody specific for the human transferrin receptor (OKT9). No expression of human transferrin receptors (M, 94,000) was observed in control Swiss 3T3 cells, but specifically immunoprecipitated human transferrin receptors were observed in fibroblast clones infected with retrovirus (Fig. 1). Expression of the murine transferrin receptor (M, 100,000) was examined by immunoprecipitation with the rat monoclonal antibodies R17217 and R17208 which bind to the murine transferrin receptor. Expression of the murine transferrin receptor was observed in control Swiss 3T3 fibroblasts and in fibroblast clones. However, the human transferrin receptor was expressed at a higher level than the murine transferrin receptor (Fig. 1) and resulted in an increased level of dipher ferr transferrin binding to the fibroblast clones (Table 1).

The transferrin receptor is a disulfide-linked dimer (33). Therefore, in fibroblast clones expressing both murine and human transferrin receptors it is possible that a mixture of murine and human homodimers and heterodimers are present. If heterodimers are expressed in the fibroblast clones, it would be expected that the human and murine transferrin receptors would be coimmunoprecipitated by monoclonal antibodies. In experiments with the monoclonal antibody OKT9, only human transferrin receptors were isolated from fibroblast clones (Fig. 1). However, when the rat monoclonal antibodies R17217 and R17208 (which bind to the murine transferrin receptor) were used an equal number of human (M, 94,000) and murine (M, 100,000) transferrin receptors were immuno-

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Regulation of transferrin receptor distribution by phorbol diesters

The distribution of transferrin receptors between the cell surface and intracellular compartments was investigated in control Swiss 3T3 fibroblasts and in fibroblast clones expressing human transferrin receptors. The fibroblast clones were incubated with 20 μg/ml of R17208. Fig. 1 demonstrates that this treatment results in the loss of murine transferrin receptors and consequently of heterodimeric receptors. The only receptor form observed in the down-regulated fibroblasts was the human homodimer, although this was expressed at a reduced level compared with untreated cells (Fig. 2). Similar results were obtained in experiments using clones expressing the Ser-24, Thr-24, and Ala-24 human transferrin receptors (data not shown). We conclude that the fibroblasts incubated with antibody R17208 are suitable for the analysis of the properties of the expressed human receptor and were used in further experiments.

Regulation of Transferrin Receptor Phosphorylation by Phorbol Diester and Growth Factors—The effect of growth factors on the phosphorylation state of the human (Ser-24) transferrin receptor expressed in Swiss 3T3 fibroblasts was examined. Treatment of the fibroblasts with PMA or PDGF caused a marked increase in the phosphorylation state of the human transferrin receptor (Fig. 3). A small increase in phosphorylation was observed when the fibroblasts were treated with EGF (Fig. 3). Dipheric transferrin was found to have no effect on the phosphorylation state of the human transferrin receptor (data not shown). Phosphopeptide mapping of the human transferrin receptor indicated that the effect of PMA, precipitated (Fig. 1). As the human receptor is expressed at a significantly higher level than the murine receptor, we conclude that the anti-murine transferrin receptor antibodies R17217 and R17208 immunoprecipitate heterodimeric transferrin receptors composed of murine and human disulfide-linked subunits. Heterodimeric murine-human transferrin receptors have also been identified by Newman et al. (34) in murine L cells transfected with human DNA.

Although human homodimeric receptors are over-expressed in the fibroblast clones, the presence of murine-human heterodimeric transferrin receptors represents a significant problem for the interpretation of the effect of specific mutations in the cDNA for the human receptor on the cycling process (Fig. 1). A strategy to decrease the expression of heterodimeric receptors was therefore employed in order to characterize the fibroblast clones obtained. The rat monoclonal antibody R17208 is an IgM which specifically inhibits the binding of diferric transferrin to the murine transferrin receptor (29). Incubation of cultured cells with anti-transferrin receptor antibodies results in the down-regulation and degradation of the transferrin receptor (35). The antibody R17208 was therefore used to down-regulate the murine transferrin receptors in fibroblast clones by incubation of the cells for 12 h with 20 μg/ml of R17208. Fig. 2 demonstrates that this treatment results in the loss of murine transferrin receptors and consequently of heterodimeric receptors. The only receptor form observed in the down-regulated fibroblasts was the human homodimer, although this was expressed at a reduced level compared with untreated cells (Fig. 2). Similar results were obtained in experiments using clones expressing the Ser-24, Thr-24, and Ala-24 human transferrin receptors (data not shown). We conclude that the fibroblasts incubated with antibody R17208 are suitable for the analysis of the properties of the expressed human receptor and were used in further experiments.

Table I

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\begin{array}{cccc}
\text{Control} & \text{PMA} \\
\text{Surface} & \text{Intracellular} & \text{Surface} & \text{Intracellular} \\
\text{cpm} & \text{cpm} & \text{cpm} & \text{cpm} \\
\hline
\text{Control Swiss 3T3 fibroblasts} & & & \\
4.20 Ser-24 & 2,401 & 49,681 & 4,799 & 47,394 \\
5.01 Ala-24 & 2,305 & 58,430 & 5,341 & 56,134 \\
3.06 Thr-24 & 2,194 & 41,716 & 4,621 & 39,217 \\
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Fig. 2. Down-regulation of murine transferrin receptors with the monoclonal antibody R17208. Control Swiss 3T3 cells and clone 4.20 Ser-24 cells that express the human transferrin receptor were metabolically labeled by incubation with [35S]methionine for 12 h. The cells were then incubated with and without 20 μg/ml of the rat IgM monoclonal antibody R17208 for 12 h. Extracts from the cells were then prepared and immunoprecipitated with antibody OKT9 to isolate human transferrin receptors (H) or with antibody R17217 to isolate murine transferrin receptors (M). Similar results were obtained in four separate experiments.
The phosphorylation state of the human transferrin receptor was then analyzed by polyacrylamide gel electrophoresis and autoradiography. Similar results were observed in three separate experiments.

Regulation of the phosphorylation state of the human transferrin receptor by growth factors. Fibroblasts expressing wild-type human transferrin receptors (clone 4.20 Ser-24) were incubated with $[^{32}P]P$ phosphate for 12 h. The antibody R17208 (20 μg/ml) was added, and the incubation was continued for a further 12 h. The cells were then treated without growth factor (lane 1) or with 10 nM EGF (lane 2), 20 ng/ml PDGF (lane 3), or 10 nM PMA (lane 4) for 5 min at 37°C. The human transferrin receptors were immunoprecipitated with the antibody OKT9, and the phosphorylation state of the receptors was then analyzed by polyacrylamide gel electrophoresis and autoradiography. Similar results were observed in three separate experiments.

PDGF, and EGF was to increase the level of two tryptic phosphopeptides (data not shown). These phosphopeptides have been previously demonstrated to be the result of incomplete trypsin digestion of the transferrin receptor, and the phosphorylated residue has been identified as serine 24 (17).

Mutation of the human transferrin receptor by substitution of alanine for serine at residue 24 also resulted in the loss of the PMA-stimulated phosphorylation of the transferrin receptor (Fig. 4). The differences in the phosphorylation of the transferrin receptor between fibroblast clones (Fig. 4) could be due to either the effect of specific mutations introduced into the receptor or to a defect in the response of the clones to phorbol diester. To resolve these possibilities the activity of protein kinase C in the fibroblast clones was examined. A similar level of expression of phorbol diester receptors and protein kinase C activity was observed in control fibroblasts and clones expressing human transferrin receptors (Table II). In order to investigate the functional activity of protein kinase C, the transmodulation of the EGF receptor caused by PMA (reviewed in Ref. 36) was examined. A similar inhibition of the binding of $^{125}$I-EGF to cell surface receptors was observed in control fibroblasts and the isolated clones (Table II). We conclude that the lack of PMA-stimulated phosphorylation of the transferrin receptor in the Thr-24 and Ala-24 clones is a result of the receptor mutations and not because of a defect in the response of the clones to phorbol diester.

Characterization of protein kinase C function and activity in Swiss 3T3 fibroblast clones

Control Swiss 3T3 fibroblasts and fibroblast clones expressing human transferrin receptors (4.20 Ser-24, 5.01 Ala-24 and 3.05 Thr-24) were investigated for the presence of functional protein kinase C activity. First, the specific binding of 5 nM $[^3H]$4β-phorbol-12,13-dibutyrate (PDBU) to cell monolayers was measured. Second, the Ca$^{2+}$ and phospholipid-dependent phosphorylation of the synthetic peptide Lys-Arg-Thr-Leu-Arg-Arg by cell extracts was examined. Third, the transmodulation of the EGF receptor in cells treated with 10 nM PMA was investigated. The effect of PMA to regulate the EGF receptor is expressed as the specific binding of $^{125}$I]potransferrin from cells. Treatment with PMA caused an increase in the first-order rate constant for $^{125}$I]potransferrin release from 0.10 min$^{-1}$ to 0.19 min$^{-1}$ (Fig. 6). Similar effects of PMA were observed on the cycling of the wild-type human transferrin receptor and receptors in which serine 24 was substituted with threonine or alanine residues (Table I, Figs. 5, and 6).
Regulation of Iron Accumulation by Phorbol Diester—The functional significance of the regulation of the cycling of the transferrin receptor in Swiss 3T3 fibroblasts was examined by investigating the rate of accumulation of radioactivity by cells incubated with 100 nM \(^{59}\)Fediferric transferrin. Treatment of control Swiss 3T3 fibroblasts with PMA increased the rate of accumulation of \(^{59}\)Fe diferric transferrin (Fig. 7). The redistribution of transferrin receptors caused by PMA is therefore associated with an increase in the rate of uptake of iron by the fibroblasts. Incubation of the fibroblasts with 20 \(\mu\)g/ml of antibody R17208 for 12 h caused a marked inhibition of iron accumulation. This result is consistent with the effect of R17208 to inhibit diferric transferrin binding to the murine transferrin receptor (29) and to cause down-regulation of the murine transferrin receptor (Fig. 2). Fibroblast clones expressing the wild-type (Fig. 7B) and mutated (Fig. 7, C and D) human transferrin receptor accumulated \(^{59}\)Fe diferric transferrin at a greater rate than control Swiss 3T3 cells. Incubation of these clones with the antibody R17208 for 12 h did not result in a significant decrease in the rate of uptake of \(^{59}\)Fe diferric transferrin (Fig. 7). Treatment with PMA caused an increase in the rate of uptake of \(^{59}\)Fe diferric transferrin in all cases. We conclude that the uptake of diferric transferrin mediated by the wild-type and mutated forms of the human transferrin receptor is regulated similarly by tumor-promoting phorbol diesters.

Effect of Growth Factors on the Cell Surface Expression of Transferrin Receptors—PDGF and EGF cause an increase in the cell surface expression of transferrin receptors in fibroblasts (9, 32). PDGF caused a marked increase in the cell surface expression of murine and human transferrin receptors in Swiss 3T3 fibroblasts (Fig. 8). EGF also increased the cell surface expression of transferrin receptors (Fig. 8), but this effect was small compared to that reported for BALB/c 3T3 fibroblasts (9). Similar results were obtained with clones expressing Ala-24 and Thr-24 human transferrin receptors (Fig. 8).

Indirect Immunofluorescence Analysis of Transferrin Receptor Expression—The distribution of transferrin receptors was examined with the antibody R17217 in control Swiss 3T3 fibroblasts treated with Triton X-100 to expose intracellular receptors (Fig. 9). Most of the murine transferrin receptors were observed to be in an intracellular juxtanuclear location.

FIG. 5. Regulation of the cell surface expression of transferrin receptors by phorbol diesters. A, the effect of PMA concentration on the binding of diferric \(^{131}\)I-transferrin to cell surface receptors. The time of PMA treatment was 10 min at 37 °C. B, the effect of the time of treatment with 10 nM PMA on the binding of diferric \(^{131}\)I-transferrin. The experiments were performed using control Swiss 3T3 fibroblasts (O) and with fibroblast clones expressing the human transferrin receptor. The fibroblast clones were incubated for 12 h with 20 \(\mu\)g/ml of antibody R17208 prior to the experiments in order to down-regulate the murine transferrin receptors. The clones used were 4.20 Ser-24 (A), 3.05 Thr-24 (▲), and 5.01 Ala-24 (●). The results are expressed relative to the binding observed in fibroblasts treated without phorbol diester (100%). Similar results were observed in four separate experiments.

FIG. 6. Regulation of the rate of transferrin receptor exocytosis by phorbol diesters. The rate of transferrin receptor exocytosis was estimated by measuring the release of \(^{131}\)I-apotransferrin from cells. The effect of treatment without (●) and with 10 nM PMA (O) at zero time is presented. A, control Swiss 3T3 fibroblasts. B, clone 4.20 Ser-24. C, clone 5.01 Ala-24. D, clone 3.05 Thr-24. The fibroblasts which express human transferrin receptors (panels B, C, and D) were incubated for 12 h prior to the assay with 20 \(\mu\)g/ml of antibody R17208 in order to down-regulate the murine transferrin receptor. Similar results were obtained in three separate experiments.

FIG. 7. Effect of phorbol diester on the rate of \(^{59}\)Fe diferric transferrin accumulation. The rate of accumulation of \(^{59}\)Fe diferric transferrin by fibroblasts treated without (△, ▲) and with (●, ○) 10 nM PMA was measured. Prior to the assay the cells were incubated without (open symbols) or with (filled symbols) 20 \(\mu\)g/ml of antibody R17208 for 12 h. The cells used included A, control Swiss 3T3 fibroblasts; B, clone 4.20 Ser-24; C, clone 5.01 Ala-24; and D, clone 3.05 Thr-24. Similar results were obtained in four separate experiments.
The cells were rapidly cooled to 0 °C and the binding of diferric transferrin to the cell surface receptors was measured. No specific binding of the monoclonal antibody R17208 which binds to the murine transferrin receptor was observed (Figs. 1 and 2). The cells obtained by this treatment expressed human homodimeric transferrin receptors (Fig. 2). The monoclonal antibody OKT9 which binds specifically to the human transferrin receptor was used to characterize the expressed human homodimeric receptors.

Regulation of the Cycling of Phosphorylation Defective Transferrin Receptors—Serine 24 is the major site on the human transferrin receptor that is phosphorylated by protein kinase C (17). Replacement of the serine residue at position 24 with alanine resulted in the loss of PMA-stimulated phosphorylation of the transferrin receptor as expected (Fig. 4). A similar result was obtained when the serine was replaced by a threonine residue (Fig. 4). This result was unexpected because the threonine substitution represents a relatively conservative mutation. Recently, House et al. (37) have investigated the substrate specificity of protein kinase C using synthetic peptides corresponding to the local phosphorylation site sequence of glycogen synthase. It was observed that the phosphorylation of the transferrin receptor expressed in Swiss 3T3 fibroblasts demonstrated that they were functional and caused the receptor-mediated endocytosis of diferric transferrin (Fig. 7). Examination of the subcellular distribution of the phosphorylation defective receptors by indirect immunofluorescence microscopy indicated no significant differences with the distribution observed for the wild-type human receptor (Fig. 9). Furthermore, the cycling of the phosphorylation-defective receptors was regulated by PMA, PDGF, and EGF in a manner similar to that observed for the wild-type human receptor (Figs. 5–8). We conclude from these results that the regulation of transferrin receptor cycling by protein kinase C is independent of receptor phosphorylation at serine 24 in Swiss 3T3 fibroblasts.

Regulation of Transferrin Receptor Cycling by Protein Kinase C—Treatment of cultured cells with tumor-promoting phorbol diesters that stimulate the activity of protein kinase C cause the phosphorylation of the transferrin receptor at serine 24 and the acute regulation of transferrin receptor cycling. In Swiss 3T3 fibroblasts (Fig. 5) and murine peritoneal macrophages (7), PMA causes a rapid increase in the expression of transferrin receptors at the cell surface. A mechanism by which PMA regulates the cell surface transferrin receptor expression in Swiss 3T3 fibroblasts is a marked increase in the rate of transferrin receptor exocytosis (Fig. 6). A functional consequence of this is an increase in receptor-mediated endocytosis of diferric transferrin resulting in a

**DISCUSSION**

**Expression of the Human Transferrin Receptor cDNA**—The strategy we have used to express the human transferrin receptor cDNA in cultured cells was to employ the retrovirus vector pZipNeoSV (X) described by Cepko et al. (25). Transfection of Psi-2 cells with plasmid constructs containing the transferrin receptor cDNA yielded stable clones resistant to G418 that provided high (>10^3 cfu/ml) titers of recombinant retrovirus. Infection of Swiss 3T3 fibroblasts with the retrovirus allowed the isolation of stable clones that express the human transferrin receptor. As the transferrin receptor is a disulfide-linked dimer, a problem encountered was the biosynthesis of heterodimeric receptors composed of murine and human monomers (Figs. 1 and 2). In order to investigate the cycling of the human homodimeric receptors, it was necessary to down-regulate the murine transferrin receptors by incubating the cells with the rat IgM monoclonal antibody R17208 which binds to the murine transferrin receptor and inhibits the binding of diferric transferrin (Fig. 2). The cells obtained by this treatment expressed human homodimeric transferrin receptors (Fig. 2). The monoclonal antibody OKT9 which binds specifically to the human transferrin receptor was used to characterize the expressed human homodimeric receptors.

Regulation of the Cycling of Phosphorylation Defective Transferrin Receptors—Serine 24 is the major site on the human transferrin receptor that is phosphorylated by protein kinase C (17). Replacement of the serine residue at position 24 with alanine resulted in the loss of PMA-stimulated phosphorylation of the transferrin receptor as expected (Fig. 4). A similar result was obtained when the serine was replaced by a threonine residue (Fig. 4). This result was unexpected because the threonine substitution represents a relatively conservative mutation. Recently, House et al. (37) have investigated the substrate specificity of protein kinase C using synthetic peptides corresponding to the local phosphorylation site sequence of glycogen synthase. It was observed that the phosphorylation of the transferrin receptor expressed in Swiss 3T3 fibroblasts demonstrated that they were functional and caused the receptor-mediated endocytosis of diferric transferrin (Fig. 7). Examination of the subcellular distribution of the phosphorylation defective receptors by indirect immunofluorescence microscopy indicated no significant differences with the distribution observed for the wild-type human receptor (Fig. 9). Furthermore, the cycling of the phosphorylation-defective receptors was regulated by PMA, PDGF, and EGF in a manner similar to that observed for the wild-type human receptor (Figs. 5–8). We conclude from these results that the regulation of transferrin receptor cycling by protein kinase C is independent of receptor phosphorylation at serine 24 in Swiss 3T3 fibroblasts.

**Regulation of Transferrin Receptor Cycling by Protein Kinase C**—Treatment of cultured cells with tumor-promoting phorbol diesters that stimulate the activity of protein kinase C cause the phosphorylation of the transferrin receptor at serine 24 and the acute regulation of transferrin receptor cycling. In Swiss 3T3 fibroblasts (Fig. 5) and murine peritoneal macrophages (7), PMA causes a rapid increase in the expression of transferrin receptors at the cell surface. A mechanism by which PMA regulates the cell surface transferrin receptor expression in Swiss 3T3 fibroblasts is a marked increase in the rate of transferrin receptor exocytosis (Fig. 6). A functional consequence of this is an increase in receptor-mediated endocytosis of diferric transferrin resulting in a
stimulation of iron accumulation by the fibroblasts (Fig. 7). Increased endocytosis of transferrin receptors has also been reported in experiments with HepG2 hepatoma cells (6), HL60 promyelocytic leukemia cells (3, 4, 15), and K562 erythroleukemia cells (5) treated with phorbol diester. However, in contrast to the results presented here with Swiss 3T3 fibroblasts, a decrease in the cell surface transferrin receptor expression observed (3, 6, 15). This difference in the response of cultured cells to PMA treatment suggests that the cycling of the transferrin receptor is under complex regulatory control. Several steps in the cell surface transferrin receptor endocytosis are acutely regulated in PMA-treated cells. For example PMA treatment stimulates pinocytosis and redirects the flow of intracellular pinocytotic fluid (31). Future progress toward understanding how protein kinase C regulates the cycling of the transferrin receptor will require identification of the relevant morphological compartment(s) of cycling transferrin receptors and the identification of the biochemical step(s) regulated.

Conclusions—We have tested the hypothesis that phosphorylation of the transferrin receptor by protein kinase C regulates receptor cycling in Swiss 3T3 fibroblasts. The site of transferrin receptor phosphorylation by protein kinase C has been identified as serine 24 (17). Wild-type and phosphorylation-defective transferrin receptors in which serine 24 was substituted with either an alanine or a threonine residue were expressed in Swiss 3T3 fibroblasts. The cycling of the phosphorylation-defective receptors was regulated by PMA, PDGF, and EGF in a manner similar to that observed for the wild-type receptor. We conclude that the regulation of transferrin receptor cycling by protein kinase C is independent of receptor phosphorylation at serine 24 in Swiss 3T3 fibroblasts.

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Note Added in Proof—Recently, Rothenberg et al. (Rothenberg, S., Iacopetta, B. J., and Kuhn, L. C. (1987) Cell 49, 423–431) reported that the internalization of transferrin receptors by phorbol ester unresponsive mouse L cells is not affected by mutation of codon 24 from serine to alanine.

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