Identification of Serine 24 as the Unique Site on the Transferrin Receptor Phosphorylated by Protein Kinase C


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Addition of tumor-promoting phorbol diesters to [32P]phosphate-labeled A431 human epidermoid carcinoma cells caused an increase in the phosphorylation state of the transferrin receptor. The A431 cell transferrin receptor was also found to be a substrate for protein kinase C in vitro. Tryptic phosphopeptide mapping of the transferrin receptor resolved the same two phosphopeptides (X and Y) after either protein kinase C phosphorylation in vitro or treatment of labeled A431 cells with phorbol diesters. [32P]Phosphoserine was the only labeled phosphoamino acid detected. Phosphopeptide X was shown to be an incomplete tryptic digestion product which could be further digested with trypsin to generate the limit tryptic phosphopeptide (Y). Radiossequence analysis of [32P]Phosphopeptide Y demonstrated that the [32P]phosphoserine was the second residue from amino terminus of the peptide. This receptor phosphopeptide was found to co-migrate with the synthetic peptide Phe-Ser(P)-Leu-Ala-Arg (where Ser(P) is phosphoserine) during reverse-phase high pressure liquid chromatography and two-dimensional thin layer electrophoresis and chromatography. The peptide Phe-Ser(P)-Leu-Ala-Arg is an expected tryptic fragment of the cytoplasmic domain of the transferrin receptor corresponding to residues 23–27. We conclude that the major site of protein kinase C phosphorylation of the transferrin receptor in vivo and in vitro is serine 24. This phosphorylation site is located within the intracellular domain of the transferrin receptor, 38 residues away from the predicted transmembrane domain.

The Ca2+- and phospholipid-dependent protein kinase (protein kinase C) is activated by diacylglycerol (1, 2). Addition of exogenous diacylglycerol to cultured cells causes the phosphorylation of specific target proteins such as the EGF receptor (3–5). The phosphorylation of these proteins by protein kinase C can also be caused by platelet-derived growth factor (6–10) which stimulates the hydrolysis of phosphatidylinositol 4,5-bisphosphate and results in an increase in the level of diacylglycerol (11), inositol 1,4,5-trisphosphate (12), and cytosolic free Ca2+ (13). Recently it has been demonstrated that protein kinase C is a major cellular receptor for tumor-promoting phorbol diesters which bind to protein kinase C at the diacylglycerol-binding site (for review see Ref. 14). The binding of phorbol diesters or diacylglycerol to protein kinase C results in an activation of the phosphotransferase activity of the enzyme (1, 2, 15) and the association of cytosolic protein kinase C with the inner surface of the plasma membrane (3, 16).

The association of protein kinase C with the plasma membrane suggests that membrane proteins are important substrates for protein kinase C. Furthermore, experiments demonstrating that phorbol diesters perturb the signaling by a number of growth factors and hormones (7, 17–20) suggest that receptors and the proteins involved in transmembrane signal transduction are physiologically relevant substrates for protein kinase C. It is, therefore, of interest that receptors such as the EGF receptor (21–26), type I insulin-like growth factor receptor (27), insulin receptor (20, 27), interleukin-II receptor (28), transferrin receptor (29, 30), β-adrenergic receptor (17), and α-adrenergic receptor (31) have been found to become phosphorylated after the addition of phorbol diester tumor promoters to intact cells. In addition two membrane proteins have been identified as substrates of protein kinase C that may be involved in signal transduction mechanisms: the α subunit of N1 (inhibitory guanyl nucleotide-binding regulatory protein) (32) and pp60c-src (33, 34).

Several studies have been reported using synthetic peptide substrates and protein substrates in order to investigate the substrate specificity of protein kinase C (35–37). These experiments have indicated that protein kinase C has a requirement for basic residues close to the phosphorylation site in the primary structure of the substrate. However, there is little information available concerning the structural requirements of membrane proteins for phosphorylation by protein kinase C. This is because the protein kinase C phosphorylation sites of only two membrane proteins have been identified. These are threonine 654 in the EGF receptor (22, 25) and serine 12 in pp60c-src (34). Gould et al. (34) have noted that there is an intriguing similarity between threonine 654 in the EGF receptor and serine 12 in pp60c-src. If it is assumed that the myristylated amino-terminal glycine residue of pp60c-src is embedded in the membrane, then the distance between the membrane and the protein kinase C phosphorylation site (9 amino acids) is the same in both proteins. This suggests that the proximity of a potential phosphorylation site to the

Received for publication, February 10, 1986


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plasma membrane surface may be an important factor in determining the specificity of protein kinase C. This hypothesis is an attractive one because of reports that indicate that protein kinase C binds strongly to membranes when activated by diacylglycerol or tumor promoters (3, 14, 16). Furthermore, the hypothesis suggests that the plasma membrane may be a fundamental component of the interaction of protein kinase C with membrane proteins.

The purpose of the experiments described in this report was to investigate whether the plasma membrane surface location of the protein kinase C phosphorylation sites on the EGF receptor and pp60^Src are similar to the phosphorylation sites on other membrane proteins. The approach that we took was to investigate the phosphorylation of the transferrin receptor by protein kinase C. The primary structure of the human transferrin receptor has recently been deduced from the cDNA sequence (38, 39). Inspection of the predicted primary structure indicated that there is a potential phosphorylation site (serine 63) located close to the predicted cytoplasmic surface of the plasma membrane in a highly basic region of the receptor (Lys-Pro-Lys-Arg-Cys-Ser-Gly). However, radiochemical sequence analysis and comparative phosphopeptide mapping demonstrated that serine 63 was not a major substrate for protein kinase C. The protein kinase C phosphorylation site was localized to a region of the receptor that is 38 residues distant from the cytoplasmic surface of the plasma membrane in the predicted primary structure of the receptor.

**EXPERIMENTAL PROCEDURES**

**Materials**—[32P]Phosphate (carrier free) and [γ-32P]ATP were from New England Nuclear and Amersham Corp., respectively. PMA and leupeptin were obtained from Sigma. Goat anti-mouse Ig was from Cappel. Monoclonal antibody OKT9 was purified from ascites fluid produced by hybridoma cells (American Type Culture Collection). Protein kinase C was prepared from bovine retinae as described in the Methods. The transferrin receptor monoclonal antibody (OKT9) was a gift from D. Massague, University of Massachusetts, New York Medical Center.

**Cell Culture and Preparation of Plasma Membranes**—A431 human epidermoid carcinoma cells obtained from Dr. G. Todaro (Oncogen) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% calf serum. Membranes were prepared by washing the cells with 120 mM NaCl, 6 mM KCl, 1 mM MgCl2, 5 mM EGTA, 25 mM HEPES (pH 7.4) and resuspending the cells in 200 mM sucrose, 5 mM EGTA, 50 mM NaF, 10 μg/ml leupeptin, 1 mM phenylmethylsulfon fluoride, 50 μM Na3VO4, 25 mM HEPES (pH 7.4) before homogenization. The homogenate was centrifuged at 10,000 X g for 10 min to give a supernatant which was layered onto a 50% (w/v) sucrose cushion. Membranes were collected by the interface after centrifugation at 90,000 X g for 90 min in a Beckman SW 41 rotor, washed twice with 25 mM HEPES (pH 7.4), and stored at -70 °C.

**Analysis of the Phosphorylation State of the Transferrin Receptor in Intact A431 Epidermoid Carcinoma Cells**—A431 cells were seeded in 35-mm dishes and grown to a density of 2 X 10^5 cells/well. The monolayers were then washed and incubated with 1 ml of phosphate-free Dulbecco’s modified Eagle’s medium supplemented with 0.1% calf serum and 3 mM/ci[32P]Phosphate. To achieve isotopic equilibrium, the cells were incubated for 24 h. The cells were then washed with and without PMA for 30 min, and the transferrin receptors were alkylated by adding 40 μl of 0.4 M iodoacetamide, 0.25 M Tris-HCl (pH 8.8) and incubation at room temperature for 15 min. Subsequently, 80 μl of 75% glycerol, 25% 2-mercaptoethanol was added, and the sample was heated to 60 °C for 15 min. After dialyzing against 0.1 M Tris-HCl, the gel slice containing the transferrin receptor was excised. The receptor was eluted with 0.1 M Na3SO4 and precipitated with trichloroacetic acid as described (41). The sample was then digested with 1 μg of tosylphenylalanyl chloromethyl ketone-treated trypsin in 100 mM N-ethylmorpholine (pH 8.0). After 5 h, a second addition of trypsin was made, and the incubation was allowed to proceed for a further 19 h.

Two methods were used to separate the complex mixture of peptides that were obtained after tryptic digestion of the transferrin receptor. The first method was two-dimensional thin layer separation on 100-μm cellulose plates (Machery-Nagel) by electrophoresis in 0.5 M acetic acid for 30 min and electrophoresis in 0.5 M acetic acid and using water butan-1-ol/pyridine/acetetic acid (60:75:50:15) as solvent. The second method used to separate the peptides was reverse-phase HPLC employing a Waters Bondapak C18 column equilibrated with 0.1% trifluoroacetic acid. After injection of the sample, the column was eluted with 5 min of solvent A (0.1% trifluoroacetic acid in 50% acetonitrile) and a linear gradient of acetonitrile (0-60%) over 60 min. The flow rate was 1 ml/min. Fractions eluted from the column were collected at 30-s intervals. [32P]Phosphopeptides were detected by Cerenkov counting.
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Phosphoamino Acid Analysis—Phosphoamino acid analysis of the transferrin receptor was performed by partial acid hydrolysis (1 h at 100 °C in 6 M HCl) and thin layer electrophoresis by the method of Hunter and Sefton (42) as described (21).

Automated Amino-terminal Sequence Analysis—Sequence analysis of [32P]phosphate-labeled peptides was performed in the presence of 4 nmol of myoglobin using a modified Beckman 890C liquid-phase sequenator and a 0.1 M Quadrupole Program (Beckman 121078). Two precolumns were performed prior to the first cleavage. The anilinothiazolines were converted to phenylthiohydantoins by reaction in 25% trifluoroacetic acid at 96 °C and were identified and quantitated by a modification of the reverse-phase HPLC procedure described by Zimmermann et al. (43) using acetonitrile. The radioactivity associated with the phenylthiohydantoins derived from the peptide that were released at each cycle was measured by liquid scintillation counting.

RESULTS

In order to compare the phosphorylation of the receptors for transferrin and EGF by protein kinase C, it is important that similar conditions are used for the experiments. Previous studies have demonstrated that the transferrin receptor of HL60 promyelocytic leukemia cells is a substrate for protein kinase C (29, 30). However, as these cells do not express EGF receptors2 we chose to investigate the phosphorylation of the transferrin receptor by protein kinase C in A431 epidermoid carcinoma cells, which we (22) and others (25) have used to identify the protein kinase C phosphorylation site on the EGF receptor (threonine 654). In preliminary experiments, the phosphorylation of the A431 cell transferrin receptor in vitro and in vivo by protein kinase C was investigated.

The ability of protein kinase C to phosphorylate the transferrin receptor was investigated by incubating plasma membranes isolated from A431 cells with a highly purified preparation of protein kinase C and [γ-32P]ATP. The phosphorylation state of the transferrin receptor was then subsequently assayed by immunoprecipitation with a monoclonal antibody (OKT9), polyacrylamide gel electrophoresis, and autoradiography. The transferrin receptor was found to have an apparent molecular weight of 180,000 when electrophoresed under nonreduced conditions, but migrated as a protein with Mr = 94,000 after reduction with dithiothreitol (Fig. 1). These observations are consistent with reports that have demonstrated that the transferrin receptor is a disulfide-linked dimer consisting of two polypeptide chains (44). We observed that there was a Ca2+-dependent phosphorylation of the transferrin receptor in the presence of protein kinase C (Fig. 1). We conclude that the A431 cell transferrin receptor is a substrate for phosphorylation by protein kinase C. A similar Ca2+ dependence of the phosphorylation of the EGF receptor in A431 membranes (24) at threonine 654 (22, 25) has been previously reported.

In further experiments we investigated whether protein kinase C could phosphorylate the transferrin receptor in intact A431 cells that had been incubated for 24 h in medium containing [32P]phosphate. To stimulate protein kinase C in intact cells we employed the potent phorbol ester tumor promoter PMA which has been shown to stimulate protein kinase C in vitro (15) and to cause the phosphorylation of proteins by protein kinase C in intact A431 cells (21–26). We observed that the addition of PMA to A431 cells caused a large increase in the phosphorylation state of the transferrin receptor (Fig. 2). This observation suggests very strongly that protein kinase C can phosphorylate the transferrin receptor in intact A431 cells. However, to substantiate this conclusion it was necessary to demonstrate that the same site(s) on the transferrin receptor was phosphorylated in these in vitro and in vivo experiments (Figs. 1 and 2). We, therefore, investigated

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Fig. 1. Phosphorylation of the transferrin receptor in vitro by protein kinase C. A431 membranes (50 μg) were incubated with a purified preparation of protein kinase C (40) in the presence and absence of CaCl2. Phosphorylation was initiated by the addition of [γ-32P]ATP (50 μCi/nmol) to a concentration of 5 μM. The reaction was allowed to proceed for 5 or 10 min at 22 °C. The transferrin receptors were then immunoprecipitated, and the phosphorylation state of the receptors was analyzed by polyacrylamide gel electrophoresis in the presence of 0.1% NaDodSO4. Reduction of the transferrin receptors with 50 mM dithiothreitol (DTT) caused the [32P]phosphate-labeled receptors to migrate with an apparent Mr = 94,000. Under nonreducing conditions, the transferrin receptor was observed to migrate as a protein of Mr = 180,000. The figure presents an autoradiograph of a dried gel. Similar results were obtained in five separate experiments.

Fig. 2. Effect of PMA on the phosphorylation state of the transferrin receptor in intact A431 cells. A431 cells were labeled with [32P]phosphate for 24 h and then treated with and without 10 nM PMA for 30 min. After solubilization of the cells, the transferrin receptors were isolated by immunoprecipitation. The immunoprecipitates were then analyzed by polyacrylamide gel electrophoresis. Reduction of the transferrin receptors with 50 mM dithiothreitol (DTT) caused the [32P]phosphate-labeled receptors to migrate with an apparent Mr = 94,000. Under nonreducing conditions the transferrin receptor was observed to be a protein of Mr = 180,000. The figure presents an autoradiograph of a dried gel. Similar results were obtained in ten separate experiments.

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Fig. 3 shows the results of two-dimensional phosphopeptide mapping of the transferrin receptor. It was found that phorbol esters caused the specific phosphorylation of the transferrin receptor on two tryptic phosphopeptides (designated x and y in Fig. 3). Similarly protein kinase C was found to phosphorylate two phosphopeptides on the transferrin receptor in isolated A431 cell plasma membranes. These two phosphopeptides were identified as phosphopeptides X and Y by mixing experiments. We conclude that PMA causes the phos-
peptide X was observed if the trypsin digestion was performed was contaminated with other proteases. In control experi-
tide X. This result suggests that either phosphopeptide X phophorylation of the transferrin receptor in intact cells on the bating the phosphopeptide with trypsin. In the presence of a were also characterized by radiochemical sequence analysis by purifying phosphopeptide X by HPLC (Fig. 3). The phosphopeptides also found that a larger amount of phosphopeptide phophopeptide Y is derived from phosphopeptide X by amont of phosphopeptide Y and a decreased level of phospho-
the greatly increased hydrophobicity of phosphopeptide X. This result suggests that either phosphopeptide X contains a poor trypsin cleavage site or that the trypsin used digestion of the transferrin receptor by high pressure liquid chromatography. A431 cells were labeled for 24 h with [32P]phospho-
peptide X after 15 cycles of degradation. However, if phosphopeptide X was first digested with trypsin prior to analysis, a peak of radioactivity was observed at cycle 2. Similarly, analysis of phosphopeptide Y also resulted in the observation of a peak of radioactivity in cycle 2. This is consistent with the finding that phosphopeptide Y is the proteolytic digestion product of phosphopeptide X. To account for the data, phosphopeptide X must contain at least an additional 14 amino acids at the amino terminus of phosphopeptide Y. This is consistent with the greatly increased hydrophobicity of phosphopeptide X compared with phosphopeptide Y as evidenced by the reten-
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![Phosphoamino acid analysis of the transferrin receptor.](image)

**Fig. 5. Phosphoamino acid analysis of the transferrin receptor.** A, phosphoamino acid analysis of transferrin receptors isolated from [32P]phosphate-labeled A431 cells treated for 30 min at 37 °C without and with 10 nM PMA or 10 nM 4P-phorbol (1, 2, and 3, respectively) was performed by partial acid hydrolysis and thin layer electrophoresis. The positions of phosphoamino acid standards are indicated. B, phosphoamino acid analysis of transferrin receptors phosphorylated in vitro by protein kinase C in the presence and absence of Ca2+. Thr(P), phosphothreonine; Tyr(P), phosphotyrosine.

**Table I**

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Radioactivity release (cpm)

Seven peptides were identified, and these are presented in Table II. One peptide (designated A in Table II) is located within the predicted intracellular domain of the receptor, one peptide (B) consists of the entire putative transmembrane domain of the receptor, and five of the peptides (C–G) are located within the extracellular domain of the receptor (Table II).

The intracellular and transmembrane locations of peptides A and B (Table II) suggested that one of these peptides might represent the peptide that is phosphorylated by protein kinase C. The residues predicted to be the phosphorylation site are serine 24 (peptide A) and serine 63 (peptide B). To investigate these predictions we prepared synthetic peptides corresponding to the regions surrounding serine 24 and serine 63 in the primary sequence of the transferrin receptor. These peptides correspond to residues 20–27 and 58–78 of the transferrin receptor. The large peptide 58–78 was found to be very insoluble because of its extremely hydrophobic nature. A shorter peptide corresponding to residues 56–66 was, therefore, prepared and used for further analysis. The structures of these three peptides are: Tyr-Thr-Arg-Phe-Ser-Leu-Ala-Arg (residues 20–27), Lys-Pro-Lys-Arg-Cys-Ser-Gly-Ser-Ile-Cys-Tyr-Gly-Thr-Ile-Ala-Val-Ile-Val-Phe-Leu (resides 58–78), and Val-Thr-Lys-Pro-Lys-Arg-Cys-Ser-Gly-Ser-Ile (residues 56–66).

The ability of two of the synthetic peptides (corresponding to transferrin receptor residues 20–27 and 56–66) to serve as substrates for protein kinase C was assayed in the presence and absence of Cu2+ and phosphatidylserine (Table III). Both peptides were observed to be phosphorylated in a Ca2+- and phospholipid-dependent manner (Table III). The peptide corresponding to residues 20–27 was a significantly better substrate than the peptide corresponding to residues 56–66. For comparison a peptide corresponding to the protein kinase C phosphorylation site (22, 25) on the EGF receptor (Lys-Arg-Thr-Leu-Arg-Arg) was also used (Table III). It was found that the transferrin receptor synthetic peptide corresponding to residues 20–27 was phosphorylated to an extent that was similar to that observed with the synthetic peptide based on the EGF receptor phosphorylation site (threonine 654). However, the synthetic peptide corresponding to residues 56–66 of the transferrin receptor was phosphorylated at a rate that was less than one-hundredth of that observed with the other peptides (Table III).

The phosphorylated synthetic peptides were further characterized by phosphoamino acid analysis and radiochemical sequence analysis. [32P]Phosphoserine was the only phosphoamino acid detected in the peptides corresponding to residues 20–27 and 56–66 of the transferrin receptor (data not shown). Radiochemical sequence analysis indicated that the major phosphorylation sites in these peptides were serine 24 and serine 65 (Table III). This data indicates that the peptide corresponding to residues 20–27 is phosphorylated on a site (serine 24) that is consistent with the site phosphorylated on the transferrin receptor by protein kinase C (Table II). However, the phosphorylation of the peptide corresponding to residues 56–66 is at a site (serine 65) that is not consistent with the site phosphorylated on the transferrin receptor by protein kinase C. This is because serine 65 is not located in a predicted tryptic peptide as the second residue from the NH2 terminus (Table II).

The availability of a synthetic peptide corresponding to part of the intracellular domain of the transferrin receptor that was phosphorylated on serine 24 (Table III) allowed us to test directly the hypothesis that the protein kinase C phosphorylation site on the transferrin receptor is serine 24.
The two phosphopeptides co-migrated during reverse-phase trypsin digestion of the synthetic peptide and phosphopeptide Tyr-Thr-Arg-Phe-Ser(P)-Leu-Ala-Arg with [32P]phosphate and treated with PMA. Fig. 6 shows that residues 20-27 of the transferrin receptor. Initial attempts to cleave the receptor only after lysine and arginine residues. Peptides containing a serine residue in the second position are presented. Five of these peptides are located in the extracellular domain of the transferrin receptor. One peptide is located in the intracellular domain of the receptor and one peptide corresponds to the predicted transmembrane domain of the receptor.

**TABLE I**

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<tr>
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<td>CSGSICYGTIAVIVFLIGMGYLYGCK</td>
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<td>C</td>
<td>131-134</td>
<td>LSEK</td>
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<td>496-508</td>
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<tr>
<td>G</td>
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**TABLE II**

Predicted tryptic peptides derived from the transferrin receptor that have a serine residue in the second position.

The primary structure of the transferrin deduced from the cDNA sequence (20, 21) was used to predict the peptides that would be obtained after trypsin digestion by assuming that trypsin will cleave the receptor only after lysine and arginine residues. Peptides containing a serine residue in the second position are presented. Five of these peptides are located in the extracellular domain of the transferrin receptor. One peptide is located in the intracellular domain of the receptor and one peptide corresponds to the predicted transmembrane domain of the receptor.

**DISCUSSION**

The protein kinase C phosphorylation site on the EGF receptor has been identified as threonine 654 (22, 25) which is located in a highly basic region of the receptor close to the predicted transmembrane domain. A similar highly basic region adjacent to the transmembrane domain of many integral membrane proteins has been reported. These basic regions are thought to be involved in the "stop-transfer" signal during the biosynthesis of membrane proteins (45, 46). Potential protein kinase C phosphorylation sites that are similar to the EGF receptor threonine 654 are present in many proteins, for example: serine 247 and threonine 250 in the interleukin-II receptor (47, 48); serine 312 and serine 315 in the class I HLA antigens (49, 50); serine 63 in the transferrin receptor (38, 39); and threonine 685 in the c-erbB2 gene product (51). In addition it has been reported that the protein kinase C phosphorylation site on pp60^c-src is in a highly basic region that may be located close to the cytoplasmic surface of the plasma membrane (34) and is homologous to the protein kinase C phosphorylation site on the EGF receptor. It is, therefore, possible that the protein kinase C phosphorylation sites on other membrane proteins may be similar to the EGF receptor threonine 654.

We have investigated the phosphorylation of the transferrin receptor by protein kinase C. This receptor contains a poten-

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[^2]: It has been previously observed that phosphorylation of a protein close to a site of trypsin cleavage can render that site insensitive to trypsin digestion (55).

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9039
A. PEPTIDE Y

B. SYNTHETIC PEPTIDE

C. MIX

FIG. 6. Comparative phosphopeptide mapping of [32P]phosphopeptide Y and a synthetic [32P]phosphopeptide (Phe-[32P]Ser(P)-Leu-Ala-Arg). A. [32P]phosphopeptide Y; B, synthetic peptide (Phe-[32P]Ser(P)-Leu-Ala-Arg); C, mix of A and B. [32P]Phosphopeptide Y was purified by reverse-phase HPLC of a trypsin digest of transferrin receptors isolated from A431 cells that had been labeled with [32P]phosphate and treated with 10 nM PMA for 30 min. To prepare the synthetic peptide, the peptide Tyr-Thr-Arg-Phe-Ser-Leu-Ala-Arg was phosphorylated with [γ-32P]ATP by protein kinase C. The phosphorylated peptide was purified by Dowex 1 chromatography and reverse-phase HPLC. The peptide was then lyophilized and subsequently digested with 1% (w/v)trypsin for 24 h at 37°C in 100 mM N-ethylmethylpholine, pH 8. The limit trypsin digestion product Phe-[32P]Ser(P)-Leu-Ala-Arg was then isolated by reverse-phase HPLC.

A potential phosphorylation site (serine 63) that is homologous to the EGF receptor threonine 654 because it is located in a highly basic region (Lys-Pro-Lys-Arg-Cys-Ser) that is adjacent to the transmembrane domain of the receptor. Digestion of the phosphorylated transferrin receptor with trypsin yielded two major phosphopeptides that were phosphorylated by protein kinase C in vitro. The two phosphopeptides were found to be related in structure and to be the result of incomplete trypsin digestion. Analysis of the phosphopeptides by partial acid hydrolysis, radiochemical sequencing, and comparative phosphopeptide mapping with synthetic peptides demonstrated that the major protein kinase C phosphorylation site on the transferrin receptor is serine 24. We conclude that the potential phosphorylation site (serine 63) that is homologous to the EGF receptor threonine 654 is not the major substrate for protein kinase C.

Inspection of the primary structure of the transferrin receptor indicates that the protein kinase C phosphorylation site (serine 24) is not located close to the transmembrane region of the receptor (38, 39). However, it is possible that the tertiary structure of the transferrin receptor is arranged so that serine 24 is located close to the cytoplasmic surface of the plasma membrane. We conclude that the protein kinase C phosphorylation site on many integral membrane proteins may not have a primary structure that is homologous to the protein kinase C phosphorylation site on the EGF receptor (threonine 654). However, the hypothesis that similarity may exist in the tertiary structure of integral membrane proteins around the protein kinase C phosphorylation site remains to be tested.

May et al. (29, 30) have suggested that the phosphorylation of the transferrin receptor by protein kinase C is important for the regulation of the cycling of the transferrin receptor. It has been reported that in different cell types tumor-promoting phorbol diesters can cause internalization (29, 30, 52, 53) or externalization (54) of the transferrin receptor. Identification of the protein kinase C phosphorylation site on the transferrin receptor will allow the hypothesis that receptor phosphorylation is directly involved in the regulation of the transferrin receptor to be tested directly. Site-directed mutagenesis of the transferrin receptor at serine 24 will provide evidence to indicate whether this amino acid is essential for the regulation of the transferrin receptor by phorbol diesters or by physiological agents that stimulate protein kinase C. These experiments are currently in progress.

Acknowledgments—We thank Dr. T. Hunter for useful discussions during the course of this work. John Cruz, Lori Kuck, and Mark Faucher are thanked for expert technical assistance. Dr. J. Massagué is thanked for assistance in the synthesis of synthetic peptides. The excellent secretarial work of Mary Halley, Judith Kula, and Karen Donahue is greatly appreciated.

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