Protein kinase C does not phosphorylate the externalized form of the transferrin receptor

Mohammed A. ADAM and Rose M. JOHNSTONE

Department of Biochemistry, McGill University, 3655 Drummond Street, Montreal, Quebec H3G 1Y6, Canada

We have investigated the phosphorylation of transferrin receptors both in intact sheep reticulocytes and in isolated plasma membranes. Phosphorylation of the receptor in intact cells or isolated plasma membranes is stimulated by phorbol diesters, suggesting that protein kinase C may be involved. Identical [^{32}P]phosphopeptide tryptic maps are formed in the presence and absence of phorbol diesters. Using heat-treated membranes (which are devoid of endogenous kinase activity), exogenous protein kinase C phosphorylates the same peptides as the endogenous kinase(s). During maturation of reticulocytes to erythrocytes, the transferrin receptor is released to the medium in vesicular form. In cells labelled with [^{32}P]P $_i$, the released receptor is not labelled with ^{32}P and the exocytosed vesicles do not phosphorylate receptor with [γ - ^{32}P]ATP. The absence of ^{32}P in the released receptor appears to be due to a change in the receptor, since, even in the presence of exogenous protein kinase C, the exocytosed receptor is phosphorylated to ~8% of the level obtained with receptors from the plasma membrane. These data suggest that during maturation and externalization the receptor is altered so that it loses its capacity to act as a substrate for exogenous protein kinase C as well as the endogenous kinase(s). This change may be a signal which segregates the receptor for externalization from the receptor pool remaining for transferrin recycling during the final stages of red cell maturation.

INTRODUCTION

The transferrin receptor from many different sources has been identified and shown to have a molecular size of 180–190 kDa, and to be composed of two identical subunits (Seligman *et al.*, 1979; Ecarot-Charrier *et al.*, 1979; Hu & Aisen, 1978; Wada *et al.*, 1979; Fernandez-Pol & Klos, 1980; Pan *et al.*, 1983). Recent studies have shown that the receptor undergoes two types of post-translational modification *in vitro*: (1) fatty acylation and (2) phosphorylation. A third modification, glycosylation, has not yet been addressed in a system *in vitro*. Receptor acylation was first demonstrated in cultured cells by Omary & Trowbridge (1981*a,b*) as an event which may occur hours after translation of the message for the receptor. Recent work in our laboratory has shown that acylation can also occur in isolated sheep reticulocyte plasma membranes (Adam *et al.*, 1984), confirming the observation that acylation can occur long after translation is complete. Phosphorylation of the receptor was first shown to occur in growing cells by Schneider *et al.* (1982), but no evidence was presented to suggest the temporal relationship between protein synthesis and phosphorylation. By showing that phosphorylation took place in isolated plasma membranes of sheep reticulocytes under conditions which did not support protein synthesis (Johnstone *et al.*, 1984), we established that phosphorylation could occur as a late post-translational event.

During maturation of sheep reticulocytes to erythrocytes, functional transferrin receptors are lost (Jandl & Katz, 1963; Kornfeld, 1969; Hemmaphard & Morgan, 1976; Frazier *et al.*, 1982; Pan & Johnstone, 1983) and mature cells are devoid of transferrin binding activity. Reticulocytes from a number of species contain from 50 000 to 200 000 transferrin receptors/cell (Frazier *et al.*, 1982; Rovera *et al.*, 1982; Iacopetta & Morgan, 1983;

Yeh *et al.*, 1982). The mechanism by which receptors are lost during maturation of sheep reticulocytes to erythrocytes has recently been shown to be associated with the formation of small vesicles bearing intact transferrin receptors which are released from the cell into the medium (Pan & Johnstone, 1983, 1984; Pan *et al.*, 1985). Harding *et al.* (1983, 1984), have also obtained similar evidence for receptor shedding via vesicle release in maturing rat reticulocytes.

The functional role of transferrin receptor phosphorylation is not known. Recent studies have shown that phorbol esters stimulate receptor phosphorylation in K562 and HL60 cells concomitant with increased internalization of the receptor (Klausner *et al.*, 1984; May *et al.*, 1984, 1985). These observations suggest a regulatory role for receptor phosphorylation. Phosphorylation does not appear to be associated with transferrin binding since the presence of the latter does not affect receptor phosphorylation in intact cells or isolated reticulocyte membranes (Johnstone *et al.*, 1984). At present there does not appear to be any chemical or physical change in the externalized transferrin receptor. Thus the receptor released from reticulocytes retains its native size and the ability to bind transferrin and anti-(transferrin receptor) antibody (Johnstone *et al.*, 1984). In addition, [^{125}I]iodotyrosyl tryptic maps of the released receptor and those of the native membrane receptor are identical (Johnstone *et al.*, 1984). In the present study we have examined the phosphorylation of the receptor by endogenous and exogenous kinases and present evidence that the released transferrin receptor is modified with respect to its ability to undergo phosphorylation. It is proposed that the change in ability to undergo phosphorylation may be a signal for sorting the receptor between the pools of receptor undergoing recycling or externalization during maturation of red cells.

MATERIALS AND METHODS

Materials

Na^{125}I , $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $[\text{P}^{32}]\text{P}_i$ were obtained from New England Nuclear. Protein A-Sepharose was a product of Pharmacia. XAR-5 film for radioautography was purchased from Kodak (Canada) Ltd. The monoclonal antibody against the transferrin receptor was obtained from Dr. J. Larrick, Cetus Corporation, Palo Alto, CA, U.S.A., and is a mouse antibody against human transferrin receptor which cross-reacts with the sheep transferrin receptor. Protein kinase C was a gift from Dr. O. M. Rosen, Sloan-Kettering Cancer Center, New York, NY, U.S.A. Protein kinase (cyclic AMP-dependent), cyclic AMP, 4β -phorbol 12β , 13α -dibutyrate, 4α -phorbol, histone type III-S, cyclic AMP-dependent protein kinase inhibitor, trypsin (TPCK-treated) and aprotinin (23 units/ml) were purchased from Sigma. Cellulose CEL-300 thin layer plates were obtained from Brinkmann, Rexdale, Ontario, Canada.

Isolation of sheep reticulocytes, plasma membranes and plasma transferrin

Reticulocytes were prepared from phlebotomized sheep and incubated *in vitro* as described (Benderoff *et al.*, 1978) using an incubation medium containing 2% calf serum. Plasma membranes were prepared by the method of Dodge *et al.* (1963), using 1/30 vol. for cell lysis. The membranes were washed twice in lysis buffer (5 mM-phosphate buffer, pH 8.0, containing 1 mM-EDTA), followed by one wash in 20 mM-phosphate buffer (pH 7.0) and resuspended in the same buffer to a final concentration of ~ 1 mg of protein/ml. Membranes were used fresh or stored frozen in buffer at -70°C . Plasma transferrin was isolated using the method described by Morgan (1964).

Phosphorylation of plasma membranes *in vitro*

A 150 μl sample of membrane suspension (~ 150 μg of protein) was incubated at 0°C in a medium (final volume 250 μl) containing 0.1 mM-EDTA, 0.1 mM-ouabain, 2 mM-dithiothreitol, 1 mM- MgCl_2 , 10 mM- NaF , 100 μM -sodium vanadate and 20 mM-phosphate at pH 7.0. The $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ concentration was 20 μM , at a specific radioactivity of 5–6 $\mu\text{Ci}/\text{mmol}$. These conditions gave the maximum amount of phosphorylation with little evidence for dephosphorylation of the ^{32}P -labelled receptor as measured by loss of ^{32}P from the receptor upon reincubation at 0°C in the absence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. To stop the reaction, 10 ml of an ice-cold solution containing 20 mM-phosphate (pH 7.0), 5 mM-EDTA and 50 mM- NaF (stopping solution) was added. No further phosphorylation was seen after the addition of the stopping solution. Initially excess non-labelled ATP was added to prevent further phosphorylation but this was found unnecessary (results not shown). The membranes were then processed for immunoprecipitation as described by Johnstone *et al.* (1984). Briefly, this involves dissolving the membranes in 1% Triton X-100 containing aprotinin, followed by immunoprecipitation at 0°C with a monoclonal antibody against the transferrin receptor and Protein A-Sepharose. Vanadate (100 μM) and NaF (10 mM) were present throughout to minimize dephosphorylation. All solutions and handling were at 0°C . The supernatants obtained after SDS extraction of the immunoprecipitates were subjected to gel electrophoresis

on 5–15% polyacrylamide gradient gels (Laemmli, 1970). For radioautography, the gels were stained with Coomassie Blue, destained, and the dry gels were exposed to Kodak X-ray film at -70°C .

$[\text{P}^{32}]\text{P}_i$ labelling of membrane proteins in reticulocytes

A 10% cell suspension ($\sim 75\%$ reticulocytes) was incubated for 3 h at 37°C in serum-free, P_i -free, Eagles' minimum essential medium containing 0.5–1.0 mCi of $[\text{P}^{32}]\text{P}_i/\text{ml}$ to equilibrate the cells with $[\text{P}^{32}]\text{P}_i$. Following preincubation with $[\text{P}^{32}]\text{P}_i$, cells were incubated for a further 30 min at 37°C without further additions, or with sheep transferrin (1 mg/ml), or anti-receptor antibody (100 $\mu\text{g}/\text{ml}$). Incubations were terminated by rapidly lysing the cells in 10 vol. of ice-cold lysing buffer, in which 10 mM- NaF and 100 mM-vanadate were present to minimize dephosphorylation. The membranes were collected by centrifugation at 30000 g for 30 mins and processed for immunoprecipitation and SDS/polyacrylamide-gel electrophoresis and radioautography as described above. For isolation of ^{32}P -labelled vesicles, long term culture was carried out as described (Benderoff *et al.*, 1978) except the medium in which the cells were incubated was essentially phosphate-free and $[\text{P}^{32}]\text{P}_i$ was added to give a final concentration of 100 $\mu\text{Ci}/\text{ml}$. Vesicles were harvested by centrifugation of the culture medium at 100000 g after removal of intact cells by centrifugation at 12000 g . The vesicles from the cultures were processed as described above (Johnstone *et al.*, 1984) to obtain immunoprecipitates of the ^{32}P -labelled transferrin receptor. Vesicles and plasma membranes were prepared for immunoprecipitation immediately after they were harvested to minimize loss of ^{32}P -labelled protein.

Phosphorylation *in vitro* of heat-treated plasma membranes and vesicle-associated transferrin receptor by purified protein kinase C and cyclic AMP-dependent protein kinase

The kinase activity responsible for endogenous transferrin receptor phosphorylation is heat labile, and incubation for 60 min at 37°C results in total loss of the kinase activity (see Figs. 2 and 3). Therefore, to use the membranes as substrate for exogenous kinases, the membranes were first incubated for 60 min at 37°C in 20 mM-phosphate buffer (pH 7.0) and then the normal incubation medium (see above) was added. The exogenous kinases used were brain protein kinase C (sp. activity 22 nmol/min per mg) or cyclic AMP-activated protein kinase (sp. activity 1.9 pM units/ μg) at 18 μg and 20 $\mu\text{g}/\text{assay}$ respectively. Cyclic AMP-dependent kinase was assayed in the presence of 20 μM -cyclic AMP. Incubations were carried out at 22°C for 30 min in a volume of 200 μl . The reactions were terminated by adding stopping solution (see above) and the immunoprecipitates were prepared as described above.

Immunoprecipitates of the transferrin receptors from membranes and released vesicles were phosphorylated by exogenous protein kinases as described for the heat-treated membranes, except that, after terminating the phosphorylation, SDS-gel sample buffer was added directly to the immunoprecipitates, the samples were heated for 5 min at 100°C and subjected to SDS gel electrophoresis and radioautographed. Quantification of the extent of phosphorylation was done by laser scanning of the Coomassie Blue stains of gels and of the radioauto-

grams to estimate protein concentration and radioactivity respectively. Alternatively, the labelled bands were eluted, digested with Protosol and counted.

Two-dimensional peptide mapping

^{32}P -labelled proteins were excised from polyacrylamide gels, digested with trypsin for 24 h at 37 °C and chromatographed on cellulose sheets according to the procedures described in detail by Elder *et al.* (1977). After separation, the labelled areas on the chromatogram were scraped off the t.l.c. plates and counted by Čerenkov radiation.

[^{125}I]Iodination of purified sheep transferrin

Iodination of the purified transferrin was carried out with chloramine-T (Hunter & Greenwood, 1962). The iodinated protein was separated from free ^{125}I by filtration on Sephadex G-25.

^{125}I -Transferrin binding in solubilized membranes

Binding of ^{125}I -transferrin to solubilized membranes were carried out as described previously by Klausner *et al.* (1983).

Dephosphorylation of the transferrin receptor and histone type III-S by vesicles

To provide a ^{32}P -labelled transferrin receptor as substrate for a putative vesicular phosphatase, reticulocyte membranes were labelled with [γ - ^{32}P]ATP by the endogenous kinase followed by immunoprecipitation of the receptor as described above. After washing the pellets three to five times with phosphate-buffered saline, the immunoprecipitates were used as substrate for dephosphorylation by the released vesicles. To provide an additional soluble substrate, histone type III-S was phosphorylated with cyclic AMP-dependent protein kinase (10 μg /assay) plus 10 μM -cyclic AMP or protein kinase C (9 μg /assay) and 20 μM -[γ - ^{32}P]ATP. The phosphorylation reaction was stopped by adding cyclic AMP-dependent protein kinase inhibitor (1 mg/assay) plus 5 mM-EDTA and 1 mM-ATP. Only EDTA and excess ATP were used to stop protein kinase C. For dephosphorylation by the released vesicles, immunoprecipitates of ^{32}P -labelled transferrin receptor or ^{32}P -labelled histone III-S were incubated with 50 μg of vesicle protein/assay for 40 min at 37 °C. Vesicles were either freeze-thawed three times or dissolved in 1% Triton X-100 prior to use to assure exposure of any cryptic phosphatases. The final volume of incubation was 250 μl . After incubation, the ^{32}P -labelled proteins were electrophoresed on SDS/polyacrylamide gels. The transferrin receptor and histone III-S bands were excised from the gel and counted by Čerenkov radiation. Histone dephosphorylation was also quantified by counting an aliquot on P-81 Whatman paper.

RESULTS

The recent demonstration that the phosphorylation of the transferrin receptor is increased by phorbol esters has suggested that protein kinase C may play a role in receptor phosphorylation and receptor translocation within the cell (May *et al.*, 1984, 1985; Klausner *et al.*, 1984; Cox *et al.*, 1985). In reticulocytes, as in growing cultured cell lines, the addition of 4 β -phorbol 12,13-dibutyrate, but not 4 α -phorbol, increases ^{32}P incorpora-

Fig. 1. ^{32}P incorporation into the transferrin receptor in intact cells

Incubation with [^{32}P]P_i was carried out as described in the Materials and methods section. The components indicated below were added after prelabelling the cells for 3 h at 37 °C with [^{32}P]P_i and incubation was continued for a further 30 min at 37 °C. Gel radioautographs of immunoprecipitates of the transferrin receptor after incubation are shown. The gels were exposed to X-ray film for 24 h at -70 °C. Lane 1, receptor phosphorylation at basal level (no additions); lane 2, sheep transferrin (1 mg/ml); lane 3, anti-receptor antibody (100 μg /ml); lane 4, cycloheximide (50 μg /ml); lane 5, 4 α -phorbol ester (100 nM); lane 6, 4 β -phorbol 12,13-dibutyrate (100 nM). The band at 186 kDa is the dimer of the transferrin receptor (TfR).

tion into the receptor by ~ 4-fold (Fig. 1, compare lanes 5 and 6).

However, it should be noted that the transferrin receptor may also be phosphorylated by other kinases, since in intact cell suspensions dibutyryl cyclic AMP at high concentration (8 mM) increases receptor phosphorylation to about the same extent as does the presence of the phorbol esters (results not shown). The effect of dibutyryl cyclic AMP on receptor phosphorylation is detectable at ~ 2 mM of the nucleotide in the medium.

Earlier work had already shown that neither anti-(transferrin receptor) antibody nor transferrin influenced receptor phosphorylation in intact cells or isolated plasma membranes. However, in the latter studies, the specific radioactivity of the cellular [^{32}P]ATP was changing during the incubation. Since differences in the specific radioactivity of [^{32}P]ATP could influence the amount of label in the transferrin receptor, the experiments were repeated under conditions of constant specific radioactivity of [^{32}P]ATP. For these experiments, the cells were incubated for 3 h with [^{32}P]P_i, prior to testing the effects of any additions on the incorporation of ^{32}P into the receptor. Control studies showed that, by 3 h, the specific radioactivity of the cellular [^{32}P]ATP was

Fig. 2. Phosphorylation of the transferrin receptor in native membranes by the endogenous kinase(s) and in heated membranes by protein kinase C and cyclic AMP-dependent protein kinase

Membranes were phosphorylated with [γ - ^{32} P]ATP and 1 mM-MgCl₂ for 30 min at 22 °C as described in the Materials and methods section. Heated membranes were prepared by preincubation at 37 °C for 60 min at pH 7.0 to inactivate endogenous kinase(s). After phosphorylation immunoprecipitates were prepared, and subjected to electrophoresis. Autoradiographs of the immunoprecipitates of the transferrin receptor after phosphorylation are shown. Lanes 1–5, all phosphorylations were carried out in presence of 100 nM-4 α -phorbol ester: lane 1, native membranes phosphorylated in absence of Ca²⁺; lane 2, native membranes phosphorylated in the presence of 1 mM-Ca²⁺; lane 3, heated membranes phosphorylated in the presence of 1 mM-Ca²⁺; lane 4, heated membranes phosphorylated in the absence of Ca²⁺; lane 5, heated membranes phosphorylated in the presence of 18 μ g of exogenous protein kinase C. Lanes 6–10, Same as lanes 1–5 respectively except 100 nM-4 β -phorbol 12,13-dibutyrate was substituted for 4 α -phorbol. Lane 11, heated membranes phosphorylated with 18 μ g of protein kinase C and 250 μ M-Ca²⁺ (compare with lane 5 for control without Ca²⁺); lane 12, as in lane 11 with the addition of 100 nM-4 β -phorbol 12,13-dibutyrate (compare with lane 10 for control without Ca²⁺); lane 13, heated membranes phosphorylated by cyclic AMP-dependent protein kinase; lane 14, as in lane 13, except that the catalytic subunit of cyclic AMP-dependent protein kinase was used.

constant (results not given). Neither the presence of transferrin, anti-(transferrin receptor) antibody nor inhibition of protein synthesis by cycloheximide affect receptor phosphorylation (Fig. 1, lanes 1–4). Although only a 30 min test period was used to examine the effects of transferrin and anti-receptor antibody, earlier studies have shown that the presence of transferrin and antibody during the entire period of phosphorylation gave the same results (Johnstone *et al.*, 1984). Moreover, the half-time for turnover of the phosphate group on the transferrin receptor is under 30 min at room temperature (Johnstone *et al.*, 1984), so that, even during a 30 min incubation period, a major portion of the pool of receptor undergoing phosphorylation would be available to the kinase(s).

As with intact cells, the presence of 4 β -phorbol 12,13-dibutyrate increases transferrin receptor phosphorylation in isolated plasma membranes (Fig. 2, lane 6). Since 4 α -phorbol is without effect on the phosphorylation of the transferrin receptor (see Fig. 1) the control phosphorylations were done in the presence of the latter. For reasons which are as yet unclear, the presence of added Ca²⁺ is not required for the phorbol ester-stimulated phosphorylation in native membranes. Indeed, the presence of Ca²⁺ may be inhibitory to phosphorylation in native membranes in the presence or absence of 4 β -phorbol 12,13-dibutyrate (Fig. 2, compare lanes 1 and 6 with 2 and 7). A similar result has been seen with other red cell systems (Palfrey & Wassen, 1985). The increase in phosphorylation by β -phorbol esters is

Fig. 3. Differential sensitivity of transferrin binding and receptor phosphorylation to heat treatment

Membrane preparations were incubated for the indicated time periods at 37 °C. Following incubation the samples were cooled to 0 °C and transferrin binding (●—●) was measured. Phosphorylation of preincubated membranes (●---●) was carried out as described for native membranes followed by SDS gel electrophoresis of the immunoprecipitates of the transferrin receptor. To quantify the extent of phosphorylation, the bands on the gel corresponding to the transferrin receptor were cut out and counted by Cerenkov radiation.

Fig. 4. Radioautograms of two-dimensional phosphotryptic peptide maps of the ^{32}P -labelled transferrin receptor

The transferrin receptor was phosphorylated in intact cells by $[\text{}^{32}\text{P}]\text{P}_i$ and with $[\gamma\text{}^{32}\text{P}]\text{ATP}$ in plasma membranes (native or heated). Incubation for 60 min at 37 °C was used to inactivate endogenous kinase(s) in membranes and either protein kinase C or cyclic AMP-dependent protein kinase was added as source of kinase. After SDS gel electrophoresis of the immunoprecipitates of the phosphorylated receptor, the latter were excised from the gels and mapped by the method described in the Materials and methods section. Electrophoresis was in the horizontal direction and chromatography was in the vertical direction. O represents the origin. (a) Intact cells labelled with $[\text{}^{32}\text{P}]\text{P}_i$ (no additions); (b) intact cells labelled with $[\text{}^{32}\text{P}]\text{P}_i$ in the presence of 100 nM-4 β -phorbol 12,13-dibutyrate (exposure time for radioautography was one-half that in (a)); (c) native plasma membranes labelled for 60 min at 0 °C with $[\gamma\text{}^{32}\text{P}]\text{ATP}$; (d) as in (c) except with 100 nM-4 β -phorbol 12,13-dibutyrate added; (e) heated membranes labelled by protein kinase C for 30 min at 22 °C in the presence of $[\gamma\text{}^{32}\text{P}]\text{ATP}$; (f) as in (e) except cyclic AMP-dependent protein kinase was used.

consistent with a role for protein kinase C in transferrin receptor phosphorylation in sheep reticulocytes. The addition of cyclic AMP or dibutyryl cyclic AMP does not influence receptor phosphorylation in isolated membranes, suggesting that the cyclic AMP-dependent kinase may be absent from the membrane preparations.

The endogenous kinase activity is not stable. Incubation of membranes for 60 min at 37 °C results in a complete loss of endogenous kinase activity (Fig. 2, compare lanes 1 and 6 with 4 and 9). However, in these preincubated membranes, the transferrin receptor will

act as a substrate for phosphorylation by exogenous protein kinase C or cyclic AMP-dependent kinase (Fig. 2, lanes 11–14). It is interesting to note that heating the membranes does not affect the ability of the receptor to bind transferrin (Fig. 3).

In contrast with the endogenous kinase, Ca^{2+} is essential for phosphorylation with added protein kinase C in absence or presence of 4 β -phorbol 12,13-dibutyrate (compare lanes 5 and 11 of Fig. 2 in the absence of 4 β -phorbol 12,13-dibutyrate with lanes 10 and 12 in the presence of 4 β -phorbol 12,13-dibutyrate). It is unlikely

Fig. 5. Phosphorylation of cell-associated and vesicle-associated (exocytosed) transferrin receptor during long term culture with [^{32}P]P $_i$

Cells were incubated with [^{32}P]P $_i$ during the entire culture period of 40 h at 37 °C. At intervals, aliquots were removed, the cells were separated from the medium and the cell-free medium centrifuged at 100 000 g for 90 min. Plasma membranes were isolated from well-washed cells, the receptor was immunoprecipitated from the Triton X-100-dissolved plasma membranes and the immunoprecipitates were subjected to SDS gel electrophoresis. The harvested vesicles were dissolved in Triton X-100, immunoprecipitated and the immunoprecipitates subjected to SDS gel electrophoresis. Note that in immunoprecipitates of the receptor from the vesicles (but not the cells) there is coprecipitation of transferrin (Tf). Since vesicles are not washed after harvesting, transferrin from the serum remains associated with the receptor and is coprecipitated with the receptor. Coprecipitation of transferrin is also obtained with cells (Pan *et al.*, 1983) unless the cells are washed extensively prior to forming the immunoprecipitates (as was done in the present experiments). (a) Coomassie Blue stains: lane 1, immunoprecipitates from the cells after 12 h incubation; lane 2, immunoprecipitates from the cells after 40 h incubation; lane 3, immunoprecipitates from vesicles collected after 12 h incubation; lane 4, immunoprecipitates from vesicles collected after 40 h incubation. (b) Radioautograms corresponding to (a). Only the receptors from the cells are highly phosphorylated. Little or no phosphorylation is seen in released receptors.

that the inhibitory effect of Ca^{2+} in native membranes is due to the activation of a proteinase which modifies the endogenous kinase(s) since (1) the effects of Ca^{2+} can be reversed by EGTA and (2) the presence of proteinase inhibitors does not affect the inhibition by Ca^{2+} (results not shown).

To determine whether the exogenous kinases phosphorylate the same peptides as the endogenous kinase(s), [^{32}P]phosphopeptide mapping was carried out. The results in Fig. 4 show that in intact cells (panel a) and isolated plasma membranes (panel c) the same four peptides are phosphorylated. Moreover, if 4 β -phorbol 12,13-dibutyrate is added to either preparation, the phosphorylation of the same four peptides is enhanced for cells and membranes respectively (panels b and d). The same four [^{32}P]phosphopeptides are also observed after phosphorylation by exogenous protein kinase C using heated membranes (panel e).

The pattern of [^{32}P]phosphopeptides formed after activation with cyclic nucleotides is different from that seen with the endogenous kinase(s) or protein kinase C. In intact cells, addition of dibutyryl cyclic AMP increases the phosphorylation of three of the four

peptides (results not shown). With exogenous cyclic AMP-dependent kinase using heated membranes, the same three peptides are phosphorylated and a new peptide (no. 5) also appears (Fig. 4f).

The similarity of the phosphopeptide maps formed by the endogenous kinase and exogenous protein kinase C supports the conclusion that both enzymes recognize the same phosphorylation sites. Although cyclic AMP-activated phosphorylation of the receptor may be physiologically significant, the sites of phosphorylation appear to be different, at least in part, from those phosphorylated by the endogenous kinase.

Since an objective of this work was to determine whether the transferrin receptor undergoes some change during maturation which may mark the receptor for externalization, it seemed pertinent to ask whether the transferrin receptor in the externalized vesicles retains the ^{32}P incorporated by the cultured cells and whether the released receptor is a substrate for exogenous kinases. For these studies, the receptor was labelled by incubating the cells at 37 °C in essentially phosphate-free medium containing [^{32}P]P $_i$. During the long-term incubation (Pan *et al.*, 1983), the excised vesicles formed were collected

Table 1. Released vesicles fail to dephosphorylate the ^{32}P -labelled transferrin receptor or histone type III-S

Transferrin receptor and histone type III-S were phosphorylated as described under 'Materials and methods'. For dephosphorylation, ^{32}P -labelled histone III-S and immunoprecipitates of ^{32}P -labelled transferrin receptor were incubated with Triton X-100-treated or thrice freeze-thawed vesicles (50 μg of protein/ml) for 40 min at 37 °C. The ^{32}P associated with transferrin receptor and histone III-S was quantified by Čerenkov radiation counting after gel electrophoresis as described under 'Materials and methods'.

Conditions	Substrate . . .	^{32}P (c.p.m.)		
		Triton X-100-treated		Freeze-thawed
		Transferrin receptor	Histone	Transferrin receptor
Control (no vesicles)		352	33 449	1496
Vesicles		376	30 784	1344
Heated vesicles (5 min at 45 °C)		392	31 910	1526

and the extent of receptor labelling in the plasma membranes and in the isolated vesicles was determined (Fig. 5). Processing of vesicles and cells was carried out in presence of NaF and vanadate to minimize dephosphorylation. It has been shown that, with time in culture, the amount of protein immunoprecipitated with anti-receptor antibody decreases in the plasma membrane and increases in the released vesicles (Johnstone *et al.*, 1984), a result consistent with the idea that the transferrin receptor protein is transferred from the cells to the vesicles.

There is detectable radioactivity in the receptor precipitated from the plasma membrane even when the amount of receptor protein is small (compare lanes 1 and 2 in Fig. 5b). In contrast, little radioactivity is found in the receptor derived from the vesicles (lanes 3 and 4 in Fig. 5b) despite the release of a large amount of receptor protein (lanes 3 and 4 in Fig. 5a). These results suggest that the released receptor is not phosphorylated. Two interpretations are possible: (1) the receptor is dephosphorylated during (or prior to) vesicle release and is fully capable of being phosphorylated by endogenous or exogenous kinases or (2) in the course of maturation, the receptor loses the ability to undergo phosphorylation. Since a receptor able to bind transferrin is released (Johnstone *et al.*, 1984) a change in the ability to undergo phosphorylation could provide a signal to discriminate between receptor designated for externalization and that which continues to recycle.

Three experiments were carried out to distinguish these possibilities: (1) the ability of vesicles to dephosphorylate ^{32}P -labelled receptor was examined; (2) the ability of isolated vesicles to phosphorylate the receptor in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ using endogenous kinase(s) and (3) the ability to phosphorylate receptor using exogenous kinases were tested. All the results are consistent with a loss of the ability to phosphorylate the receptor.

(1) Released vesicles do not dephosphorylate the ^{32}P -labelled receptor from plasma membranes (Table 1). Thus incubation of vesicles with ^{32}P -labelled receptor does not remove ^{32}P from the immunoprecipitated transferrin receptor. Immunoprecipitates of the receptor were used as substrates for dephosphorylation to enrich selectively the specific substrate. As a control,

histone III S phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was also used as substrate for the phosphatase in the vesicles. Although histone phosphorylation by cyclic AMP-dependent kinase is shown in Table 1, an identical result is obtained with histone phosphorylated by protein kinase C. There is no evidence for dephosphorylation of the phosphoprotein substrates by the vesicles. Both Triton X-100-treated and freeze-thawed vesicles were used to ensure that cryptic phosphatases on the inner surface of the vesicles would be exposed. Native vesicles also failed to dephosphorylate the receptor. Indeed, attempts to dephosphorylate the receptor with excess alkaline phosphatase also failed (results not shown).

(2) We have reported (Johnstone *et al.*, 1985) that freeze-thawed vesicles phosphorylate exogenous histones as well as a number of endogenous vesicle proteins in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The transferrin receptor, however, is not phosphorylated under these conditions.

(3) Most importantly, exogenous protein kinase C does not phosphorylate the vesicular receptor (Fig. 6). For these studies, immunoprecipitates of the transferrin receptor were used as substrates for phosphorylation for three reasons: (a) the immunoprecipitates from cells and vesicles provide the receptor as substrate to the exogenous kinases in a comparable form; (b) since the receptor in the vesicles is right-side-out (Pan *et al.*, 1985) the phosphorylation sites might not be available to the exogenous kinases in the vesicles; (c) immunoprecipitates of the transferrin receptor phosphorylated by exogenous protein kinase C yield ^{32}P phosphopeptides (nos. 1–4) comparable with those obtained with native membranes (compare Fig. 4a with Fig. 7a). A few additional, and generally minor, peptides are also phosphorylated in immunoprecipitates.

In the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and protein kinase C, immunoprecipitates of the receptor in vesicles fail to undergo significant phosphorylation whereas, under identical conditions of incubation and immunoprecipitation, the receptor from plasma membranes is well phosphorylated (Fig. 6b). It should be noted that similar amounts of immunoprecipitated receptor were used for both assays (compare lanes 1 and 2 with 3 and 4 in Fig. 6a). Freeze-thawed vesicles were also used as substrate for phosphorylation by protein kinase C to check whether the low level of phosphorylation was associated

Fig. 6. Phosphorylation of immunoprecipitated receptors from fresh membranes and exocytosed vesicles by using purified protein kinase C and cyclic AMP-dependent protein kinase

Immunoprecipitates of the transferrin receptor from plasma membranes and released vesicles were phosphorylated with exogenous kinases for 30 min at 22 °C as described in the Materials and methods section. The immunoprecipitates were subjected to SDS gel electrophoresis and radioautography. Note that in lanes 3 and 4 (panel *a*) transferrin (Tf; 80 kDa) coprecipitates with immunoprecipitates of the receptor obtained from vesicles (see legend of Fig. 5). (*a*) Coomassie Blue stain of immunoprecipitates from plasma membranes (lanes 1 and 2) and from exocytosed vesicles (lanes 3 and 4). Lane 1, immunoprecipitates from plasma membranes phosphorylated with cyclic AMP-dependent protein kinase; lane 2, immunoprecipitates from plasma membranes phosphorylated with protein kinase C; lane 3, immunoprecipitates from vesicles phosphorylated with cyclic AMP-dependent protein kinase; lane 4, immunoprecipitates from vesicles phosphorylated with protein kinase C. (*b*) Radioautograph corresponding to (*a*).

with some unknown problem of vesicular immunoprecipitates. No evidence for phosphorylation was obtained in freeze-thawed preparations of vesicles in contrast with plasma membranes (results not shown).

The possibility that the phosphorylation sites in the vesicles are inaccessible to protein kinase C was considered unlikely since an identical protocol was used with plasma membranes which undergo extensive phosphorylation. Both preparations were pretreated with Triton to obtain the immunoprecipitates. Moreover, cyclic AMP-dependent kinase, which phosphorylates some common peptides, retains ability to phosphorylate the immunoprecipitated externalized receptor. Thus the likelihood of an inaccessible site on a common peptide appears small.

Finally, it cannot be argued that vesicles contain a protein kinase C inhibitor which prevents its action on vesicular substrates since the phosphorylation of histones by protein kinase C is not affected by the presence of vesicles (results not shown). Also, the presence of transferrin does not affect transferrin receptor phosphorylation by the exogenous kinases in immunoprecipitates of the transferrin receptor. Transferrin bind-

ing is readily detected in immunoprecipitates (Fig. 6*a*).

Attempts to quantify the phosphorylation of the immunoprecipitated receptors from the vesicles by exogenous protein kinase C show that the extent of phosphorylation is ~8% of that of receptors derived from the plasma membrane (Fig. 6*b*, compare lanes 2 and 4). These studies were done by laser densitometry of the radioautographs and by counting the excised bands from gels. Exogenous cyclic AMP-dependent kinase phosphorylates the immunoprecipitated receptor from vesicles at ~77% of the level found with the immunoprecipitated receptor from the plasma membrane (Fig. 6*b*, compare lanes 1 and 3).

Although the receptor in immunoprecipitates of vesicles is poorly phosphorylated by protein kinase C compared with the receptor in immunoprecipitates of the plasma membrane, the patterns of phosphorylation are generally similar but not identical (compare Figs. 7*a* and 7*c*). It may also be seen that peptide no. 3 in immunoprecipitates of vesicles (Fig. 7*c*) is relatively less phosphorylated than peptide no. 3 from immunoprecipitates of the plasma membrane (Fig. 7*a*). The overall intensity of the peptides in Fig. 7(*c*) should not be

Fig. 7. [^{32}P]Phosphotryptic map of the transferrin receptor obtained after labelling the immunoprecipitates with exogenous kinases

(a) Immunoprecipitates of the receptor from plasma membranes phosphorylated with exogenous protein kinase C; (b) as in (a) but using exogenous cyclic AMP-dependent kinase; (c) immunoprecipitates of the receptor from released vesicles phosphorylated with exogenous protein kinase C; (d) as in (c) but using exogenous cyclic AMP-dependent kinase. The intensity of labelling in (c) should not be compared with that in (a), (b) and (d) since the exposure time for (c) was 7 times longer than for (a), (b) and (d).

Table 2. Distribution of ^{32}P in the phosphopeptides after phosphorylation by endogenous kinase, protein kinase C and cyclic AMP-dependent kinase

The experiments were conducted as described in Fig. 7. After separation of the peptides, the appropriate areas on the thin layer plate were scraped off and the radioactivity counted. The total radioactivity recovered from each plate was considered as 100%. The data are presented as percentages of total radioactivity in each of the peptides based on two experiments which agreed within 10%. Immunoprecipitates of plasma membranes were used as substrate for phosphorylation.

Phosphorylating enzyme	Peptide no. . . .	Phosphorylation in individual peptide (% of total)				
		1	2	3	4	5
Protein kinase C		30	27	30	10	—
Cyclic AMP-dependent protein kinase		20	23	20	—	37

directly compared with those in Figs. 7 (a), 7(b) and 7(d), since the total exposure time was about seven times longer for panel (c) than for panels (a), (b) and (d) in experiments executed simultaneously, under the same conditions, emphasizing again the relatively poor phosphorylation of the transferrin receptor derived from vesicles.

The [^{32}P]phosphopeptide pattern obtained after phosphorylation of immunoprecipitates with exogenous cyclic AMP-dependent kinase shows the appearance of a highly phosphorylated additional peptide (no. 5, Fig. 7b). Although the common peptides (nos. 1, 2 and 3) are phosphorylated, peptide no. 5 is prominently labelled

whereas peptide no. 4 is absent. Peptide no. 5 may account for 35–40% of the total ^{32}P incorporated (Table 2). Indeed, the appearance of this highly phosphorylated peptide in the immunoprecipitates may underestimate the loss in ability to phosphorylate the remaining peptides of the externalized receptor by cyclic AMP-dependent kinase. Based on the total radioactivity in the three common peptides, the loss in phosphorylation by cyclic AMP-dependent kinase is near 50% with respect to the phosphorylation of the receptor in plasma membranes (63% of 77%).

The overall data presented above show that a change has occurred in the released receptor such that its capacity

to act as a substrate for phosphorylation by exogenous protein kinase C or by the native kinase(s) is lost or greatly diminished, whereas the capacity to act as a substrate for cyclic AMP-dependent kinase is partly retained.

DISCUSSION

The transferrin receptor has been shown to undergo two kinds of post-translational modifications: acylation (Omary & Trowbridge, 1981a,b; Adam *et al.*, 1984) and phosphorylation (Johnstone *et al.*, 1984; May *et al.*, 1984; Klausner *et al.*, 1984; Hunt *et al.*, 1984; Cox *et al.*, 1985). The role of these modifications is not clearly understood. Recent reports (May *et al.*, 1984; Klausner *et al.*, 1984) show that the enhanced phosphorylation of transferrin receptors (mediated by phorbol diesters) stimulates their internalization in K-562 and HL-60 cells. In contrast, Cox *et al.* (1985) showed that, in rabbit reticulocytes, haemin reduces endocytosis and receptor internalization with the result that the amount of receptor available for phosphorylation on the cell surface is increased. Thus the results of Cox *et al.* (1985), which show an increase in phosphorylation of the receptor when internalization is reduced, contrast with those above, which show increased phosphorylation when internalization is increased. Despite the disparate nature of the two observations, both implicate transferrin receptor phosphorylation in signalling receptor endocytosis.

Several investigators have concluded that transferrin binding does not influence receptor phosphorylation (Johnstone *et al.*, 1984; May *et al.*, 1984). The present observations show that, in heat-treated membranes, the receptor binds transferrin normally when phosphorylation is completely abolished. The latter observation, however, does not shed light on whether a phosphorylated or unphosphorylated receptor binds transferrin, since only a very small fraction of the receptor becomes phosphorylated by [^{32} P]P_i or [γ - ^{32} P]ATP in native cells or membranes, respectively (Johnstone *et al.*, 1984). Since dephosphorylation of the receptor is not blocked by conditions which inhibit phosphorylation (i.e. 60 min incubation at 37 °C) (result not shown) and ^{32} P turnover on the transferrin receptor has a half-time of about 30 min (Johnstone *et al.*, 1984), the unaltered binding of transferrin to heat-treated membranes provides some evidence that a phosphorylated receptor is not required for transferrin binding.

We have long been puzzled by the fact that during maturation of sheep reticulocytes, the transferrin receptor is shed (in vesicular form) with a seemingly unaltered receptor (Pan & Johnstone, 1983; Johnstone *et al.*, 1984). This dilemma raised the question of the signals used to discriminate between the recycling of the receptor for iron delivery and those used to designate the receptor for externalization. The present observations provide some evidence which may reflect the nature of the signals used for discrimination. We have found that, despite an unchanged molecular size, unchanged [^{125} I]iodotyrosyl peptide map and the retention of the capacity to bind transferrin, the released transferrin receptor is no longer a substrate for phosphorylation by the endogenous kinase(s) and protein kinase C. The transferrin receptor in heated membranes, or in immuno-

precipitates from Triton X-100-dissolved membranes, is readily phosphorylated by exogenous protein kinase C. In contrast, the receptor in immunoprecipitates of vesicles is a poor substrate for protein kinase C. Moreover, it may also be significant that although the overall phosphorylation of the receptor in immunoprecipitates of vesicles is ~8% of that in immunoprecipitates from plasma membranes, the phosphorylation of [^{32} P]phosphopeptide no. 3 from vesicles (compare panels *a* and *c* in Fig. 7) is relatively more reduced than that of the other three peptides.

The change in the receptor's ability to undergo phosphorylation during (or as a result of) externalization is unlikely to be due to inaccessibility of the sites of phosphorylation, since another kinase retains ability to phosphorylate some common peptides.

With cyclic AMP-dependent kinase, an additional peptide (no. 5) becomes highly phosphorylated in immunoprecipitates of the receptor. To some extent the high level of phosphorylation of peptide no. 5 accounts for the overall similarity in the extent of ^{32} P incorporation into receptor derived from immunoprecipitates of membranes and vesicles respectively.

It is interesting to consider that, during receptor-mediated endocytosis/exocytosis of transferrin for iron delivery, the small cytoplasmic component at the *N*-terminal region of the receptor (about 5 kDa) which contains the phosphorylation sites (Schneider *et al.*, 1982), will remain exposed to the cytosol while the surface binding region (~70 kDa) is enclosed in the endosome and protected against alteration by cytosolic reactions. It is also known that the exterior-facing 70 kDa part of the receptor (the *C*-terminal end) is still capable of binding transferrin after cleavage of the receptor from the cell with trypsin (Schneider *et al.*, 1982). Thus, it is realistic to consider that a change in the ability to phosphorylate the receptor could occur without a major change in transferrin-binding activity during reticulocyte maturation. The decreased phosphorylation by exogenous protein kinase C suggests that in the vesicles there has been some conformational or structural change at the *N*-terminal phosphorylated region of the receptor. This change may be the signal for routing the receptor from the recycling path to that destined for externalization. Since a number of plasma membrane proteins appear to be externalized in the vesicles (Johnstone *et al.*, 1985), it will be interesting to determine whether other externalized proteins are structurally modified.

In this paper, we show that the phosphorylation of the transferrin receptor can be stimulated by active phorbol diesters in native sheep reticulocytes or isolated reticulocyte membranes. The activation of phosphorylation by phorbol diesters suggests that protein kinase C participates in the phosphorylation (Kikkawa *et al.*, 1983; Neidel *et al.*, 1983; Sando & Young, 1983; Castagna *et al.*, 1982). If the baseline and phorbol diester-activated phosphorylation are both due to the presence of protein kinase C in washed membranes, the behaviour of the enzyme is somewhat unusual in that added Ca²⁺ inhibits phosphorylation and pretreatment of membranes with 2 mM-EGTA/5 mM-EDTA increases receptor phosphorylation (result not shown). The inhibition by Ca²⁺ can be reversed by EGTA. Similar responses to Ca²⁺ were described by Palfrey & Wassen (1985), with red cell protein kinase C. Further experiments are required to conclude unambiguously whether basal phosphorylation

of the transferrin receptor is due to an adventitious protein kinase C or other kinase(s).

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